Evaluation of Genetic Diversity in Worldwide Germplasm Collection of Garlic (*Allium sativum* L.)

ニンニクの世界的遺伝資源コレクションにおける遺伝的多様性の評価

A thesis submitted as part fulfillment for a PhD in

AGRICULTURE

The United Graduate School of Agricultural Sciences

Tottori University

JAPAN

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2016

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Chapter 1. GENERAL INTRODUCTION

Allium is a large genus comprised of 750–780 species (Fenwick and Hanley 1985; Kamenetsky and Rabinowitch 2006). It is widely distributed, especially in the northern hemisphere. Major *Allium* crops include bulb onions (*Allium cepa* L.), garlic (*Allium sativum* L.), Japanese bunching onions (*Allium fistulosum* L.), leeks or great-headed garlic (*Allium ampeloprasm* L.), and rakkyo (*Allium chinense* G Don.).

Garlic is a widely cultivated crop, second in importance only to the bulb onion. Garlic is grown in various countries at a wide range of latitudes and with a total production of 24.8 million ton per year. China, India, the Republic of Korea, and Egypt are its principal producers (FAO 2012). China is the chief producer of garlic (about 80% of the total production); Shandong Province is the largest garlic producer (Fig. 1). Moreover, garlic has garnered much attention for being rich in chemicals. It is used both for food and for medical purposes, due to its antithrombotic, lipid-lowering cardiovascular and anticancer effects (Shenoy and Choughuley 1992; Agarwal 1996; Ruddock et al., 2005). Garlic extract has been used as a traditional medicine for the prevention and treatment of cardiovascular disease (Ackermann et al., 2001). To meet the demand for rich chemicals that play roles as antioxidants, many commercial preparations derived from garlic have been made available over the years (Gillian et al., 2006) (Fig. 2). Today, large quantities of garlic bulbs are consumed as food or for pharmaceutical purposes worldwide (Kik et al., 2001).

Fig. 1 Broad fields of garlic and onion in Shandong province, China.

Fig. 2 Various kinds of garlic health supplements in the world.

Garlic's progenitors and center of origin were uncertain until recently. Many researchers inferred that garlic's original habitat was southwestern Siberia (Etoh and Simon 2002). However, Vavilov (1951) and Kazakova (1971) proposed Central Asia as garlic's primary center of origin, with the Mediterranean and the Caucasus as secondary centers. Etoh (1985; 1986) discovered many fertile garlic clones on the northwestern side of the Tien Shan Mountains in central Asia. The progenitor species of garlic was also unclear. *Allium longicuspis* Regel, the closest relative of garlic, is morphologically or karyologically very similar to garlic (Etoh 1985). Thus, this species has been considered to be a wild race of garlic (Etoh and Simon 2002). However, this plant did not always show its fertility. McCollum (1976) described circumstantially fertile *A. longicuspis* accessions in the Central Asia region. As a result, fertile garlic was found in Kyrgyzstan (western Tien Shan), Kazakhstan (surrounding Karatau Mountain), and Uzbekistan (the Chatkal Mountains). Etoh (1986) suggested that this area may be the original habitat of garlic or the ancestor of garlic because much of the material collected there was fertile. Moreover, a more detailed survey of the origin of *A. longicuspis* was carried out that found that its natural habitat ranged from the Kopet Dag mountain range (between Turkmenia and Iran) to Tien Shan Mountain, with the Pamir-Alai Mountains in the middle (Etoh and Simon 2002). England (1991) named the area *the garlic crescent*. In addition, Mathew (1996) extended this area to eastern Turkey as the main natural distribution of garlic. Thus, this area was called *the extended garlic crescent*. *A. longicuspis* was considered to be garlic's closest wild relative, the wild ancestor of garlic (Etoh and Simon 2002). Alternatively, Etoh (1986) suggested a new possibility that *A. longicuspis* and *A. sativum* might have a common ancestor. In addition, Mathew

(1996) also suggested an interesting new possibility that *Allium tuncelianum* (*macrochaetum*), a native species in Turkey, might be the common ancestor of garlic and *A. longicuspis*. This plant had similar traits to garlic (such as several morphological characteristics and a common odor). However, later investigation demonstrated that this plant is not the immediate wild ancestral species of garlic (Ipek et al. 2008).

England (1991) proposed that wild *A. longicuspis* might have been cultivated by semi-nomadic hunter-gathers more than 10,000 years ago. Additionally, England (1991) suggested that nomadic tribes might have transported wild garlic. An ancestral garlic population in Central Asia was spread widely to various regions. The history of serious garlic cultivation goes back approximately 3,000 BC (Etoh and Simon 2002). In Egypt, clay models of garlic were discovered in predynastic Egypt more than 5000 years ago. In India, a reference to garlic was found in the ancient *Vedas* (circa 800 BC) (Khar et al. 2011). Eating *Allium* plants such as onions and garlic was believed to increase passions like anger and sex drive; therefore, eating them was considered taboo (Rochow 2009). Thus, *Allium* plants such as onions and garlic seem to have given rise to some religious laws. Garlic was introduced to Japan through Korea, where garlic has become a very popular plant; in Japan, however, it has almost never been used due to the reasons mentioned above (Etoh and Simon 2002).

For centuries, this plant has been propagated clonally, which has, perhaps, resulted in a bottleneck effect for genetic variation (Ma et al. 2009). However, cultivated garlic or clonal lineages exhibit remarkably great morphological variation in leaf number, bulb size and structure (such as arrangement and number and size of the cloves), floral scape length, and inflorescences (Pooler and Simon 1993; Keller 2002; Kamenetsky et al., 2005; Buso et al., 2008). Characterization of the garlic germplasm

has been based largely on phenotypic characteristics. However, morphological characteristics can vary under different agroclimatic conditions (Jo et al., 2012). This situation makes the characterization of garlic clones complex (Mario et al., 2008). Many researchers have studied morphological traits and molecular markers such as isozymes and DNA to evaluate the diversity of garlic (Pooler and Simon 1993; Maass and Klaas 1995; Etoh et al., 2001; Lampasona et al., 2003; Zhao et al., 2011; Jo et al., 2012). Maaß and Klass (1995) categorized garlic species into four subspecies based on morphological and isozyme variation: the *longicuspis* group, including most garlic clones from Central Asia; the *subtropical* group, which developed under the climatic conditions of Southeast and East Asia; the *ophioscorodon* group, which is derived from Eastern Europe; and the *sativum* group, which is from the Mediterranean.

It is believed that the evolution of garlic accelerated after the start of domestication followed by cultivation in various regions. Garlic lost its fertility. It is uncertain whether garlic became sterile after the beginning of its cultivation, but sterility in garlic is no doubt a consequence or a product of the species' evolution, including domestication (Etoh 1985). In studies about garlic sterility, Koul and Gohil (1970) stated that flowers and bulbils competed for nutritional resources for developing inflorescences. It has been suggested that one of the causes of sterility is that garlic produces bulbils in the inflorescence (Kamenetsky et al., 2005). According to Etoh (1985), one of the main causes of sterility in garlic must be the abnormal constitution of chromosomes. As reported by Bozzini (1991), regular garlic cultivars have a somatic chromosome number of $2n = 16$ (with a karyotypic formula of six metacentric chromosomes, four submetacentric chromosomes, and six acrocentric chromosomes). However, Etoh (1985) demonstrated the great variation in chromosome pairings at

meiosis within a species (8Ⅱ, 1Ⅵ+6Ⅱ, 1Ⅵ+ 5Ⅱ, 1Ⅷ+4Ⅱ, 1Ⅸ+3Ⅱ, and desynapsis or asynapsis). Etoh (1979) stated that chromosomal changes in garlic may tend to accumulate more than in other plants that propagate vegetatively. Extensive karyotypic evaluation by Hong et al. (2000) found the basic karyotype (two sets of chromosomes) in clones from Central Asia.

Studies of fertile garlic have been carried out by many researchers. The ability to cross garlic would bring great benefits to the field of breeding. Additionally, fast propagation of desired genotypes via true seeds would be expected to result in reduced storage costs and fewer injuries caused by viruses, diseases, and pests transmitted by infected propagules. Therefore, the restoration of fertility and of sexual reproduction would permit genetic studies and the classical breeding of garlic (Kamenetsky et al., 2005). In an effort to overcome garlic sterility, many researchers have tried to obtain true garlic seeds (Etoh et al., 1988; Pooler and Simon 1994; Hong and Etoh 1996; Hong et al., 2000; Simon and Jenderek 2003; Kamenetsky et al., 2004).

Garlic has spread to various regions through the process of domestication with accumulating mutations to adapt to different agroclimatic environments. As a result, garlic varies morphologically from region to region. Some garlic may possess superior traits, such as the high production of chemicals that contribute to human health, high tolerance to disease and pests, and high adaptation to biotic or abiotic stress, as compared to current cultivars. However, reports about the evaluation of diversity on a number of fronts (such as morphological, physiological, chemical, and genetic) characteristics in various regions of garlic have been limited.

In this study, to evaluate the diversity of garlic worldwide, we investigated the available research as follows: (1) the association between bio-morphological traits and

the geographical distribution of garlic; (2) variations in the saponin production of garlic's genetic resources; and (3) evaluation based on morphological, physiological, and isozyme variations in garlic.

This chapter is the first of five that comprise this dissertation. Chapter 2 deals with objective 1, chapter 3 refers to objective 2, chapter 4 gives information on objective 3, and chapter 5 contains a general discussion. This dissertation is a compilation of the results of studies conducted by the author at the Laboratory of Vegetable Crop Science, Division of Agrobiology, Department of Biological and Environmental Sciences, Faculty of Agriculture, Yamaguchi University, Japan, with the above objectives from 2010 to 2015 (Hirata et al. 2015a; 2015b).

Chapter 2. CHARACTERISTICS OF CHEMICAL COMPONENTS IN GENETIC RESOURCES OF GARLIC *ALLIUM SATIVUM* COLLECTED FROM ALL OVER THE WORLD

Introduction

Garlic (*Allium sativum* L.) has been used since ancient times as a spice, condiment, vegetable, and medicine. According to the United Nations Food and Agriculture Organization (FAO 2012), garlic is the second most widely cultivated member of the *Allium* species, after onions. Today, this vegetable is cultivated in many places around the world.

Garlic is usually a sterile plant and is propagated vegetatively by cloves or bulbils. Nutritional competition has occurred between flowers and bulbils in developing inflorescences, since they are usually produced in the inflorescence (Koul and Gohil 1970). It has been suggested that one of the main causes of sterility is that garlic produces bulbils in the inflorescence (Kamenetsky et al. 2005). The center of origin for garlic is considered to be the northwestern side of the Tien Shan Mountains, Central Asia, because a number of fertile clones of a primitive garlic type were discovered in this area (Etoh and Simon 2002). Breeding studies to obtain true garlic seeds have been attempted by many researchers (e.g., Etoh et al., 1988; Pooler and Simon 1994; Hong and Etoh 1996; Hong et al., 2000a; and Kamenetsky et al., 2004).

Garlic clones vary not only in their fertility but also in most vegetative characteristics, such as leaf number, bulb size and structure, floral scape length, and

inflorescence development (Kamenetsky et al., 2005). In bolting traits, the number of bulbils and their size vary with genotype (Etoh 1985; 1986). Etoh (1985) reported garlic bolting habits as follows: (1) Complete bolting: plant always bolts, and its flower stalk fully elongates high above the ground. The inflorescences come out of the leaf sheaths. (2) Incomplete bolting: plant produces a thin, short flower stalk and bears only a few bulbils in the leaf sheaths. (3) Non-bolting: plants neither bolt nor develop flower buds.

Garlic contains a wide range of chemicals, such as sulfur compounds (Kamenetsky et al., 2005; Hornickova et al., 2009) and phenolic compounds (Lu et al. 2011), which have benefits for human health. They contribute to lower total plasma cholesterol, reduce blood pressure, and decrease platelet aggregation (Sterling and Eagling 2001). Both sulfur and phenolic compounds contribute to antioxidant activity in *Allium* species. Most of medical effects of garlic are attributable to sulfur compounds. *Allium* species contain different kinds and levels of sulfoxides, such as AlCSO (S-allyl-L-cysteine sulfoxide), MeCSO (S-methyl-L-cysteine sulfoxide), and PRENCSO (S-1-propenyl-L-cysteine sulfoxide) (Kyung 2012). AlCSO, known as alliin, did not exist in other major *Allium* species, such as the bulb onion or shallot (*A. cepa*) and the Japanese bunching onion (*A. fistulosum*) (Yoo and Pike 1998). Phenolic compounds are products of the secondary metabolism of plants and are largely influenced by genetic factors and environmental conditions (Bravo 1998). They are known as antioxidant phytochemicals, and studies of the phenolic compound content of garlic for antioxidant activity have been carried out by several researchers (Lu et al., 2011; Chen et al., 2013).

Although garlic is produced in a wide geographic range, there have been few studies on the chemical compounds in garlic based on geographical origin, and little is

known about their similarities and differences. Therefore, the objective of this chapter was to determine some chemical compounds in garlic bulbs for each collected area and to perform research to show the association between biomorphological traits and geographical distribution.

Materials and Methods

Plant materials

Bulbs of 103 garlic accessions have been collected from around the world since the 1970s and were managed at Kagoshima University, Japan (31.56 °N, 130.54 °E), until 2008, when management of these collections was taken over by Yamaguchi University, Japan (34.14 °N, 131.47 °E). Seventy-five accessions from Asia (26 accessions from Japan, 8 accessions from China, 9 accessions from a tropical-subtropical area, and 32 accessions from Central Asia), 22 accessions from Europe (14 accessions from the Northern Mediterranean and 8 accessions from the Southeast Mediterranean), 3 accessions from the New World area, and 3 accessions from unknown areas were used in this study (Table 1). These bulbs were obtained from local markets or national institutions in each country. Some collected accessions have detailed information and are shown by Etoh (1985), Etoh (1986), Hong et al. (2000a), and Etoh et al. (2001) (Table 1). These bulbs were stored at 4℃ in dark conditions in the summer.

The garlic collection was planted in an experimental field at Yamaguchi University in October 2008. For each accession, eight cloves were planted. The row and plant-to-plant spacing were 20 cm, and the depth of the seeding furrow was 10 cm. All bulbs were harvested in June and July 2009.

Table 1. Garlic accessions used in this chapter.

Accession number	Collected country or site	Accession information	Remarks column	Year introduced to Kagoshima, Japan
5	Japan	Etoh 1985	"Howaito-Roppen"	1972
6	Japan	Etoh 1985	"Niigata-Sado"	1972
8	Japan	Etoh 1985	"Ibaraki"	1972
9	Japan	Etoh 1985	"Chiba-A"	1972
14	Japan	Etoh 1985	"Chiba-B"	1972
15	Japan	Etoh 1985	"Hamamatsu"	1972
16	Japan	Etoh 1985	"Wakayama-Roppen"	1972
32	Japan	Etoh 1985	"Iki-No. 1"	1972
37	Japan	Etoh 1985	"Okute-B"	1972
39	Taiwan	-	"Seira"	1972
40	Japan	Etoh 1985	"Kokotsu"	1972
44	Taiwan	Etoh 1985	"Taiwan-daikyu-pinku"	1972
45	Taiwan	Etoh 1985	"Taiwan-shokyu-pinku"	1972
54	China	Etoh 1985	"Fukushu (Foochow,China)"	1972
55	Egypt	Etoh 1985	"Egypt"	1972
56	Japan	Etoh 1985	"California Early"	1972
60	Chili	Etoh 1985	"Chili"	1972
63	Japan	Etoh 1985	"Saga-zairai"	1972
64	China	Etoh 1985	"Shanhai-wase"	1972
65	Japan	Etoh 1985	"Iki-shu"	1972
67	Japan	Etoh 1985	"Amami-A"	1972
68	Japan	Etoh 1985	"Amami-B"	1972
75	Japan	Etoh 1985	"Kushikino-wase"	1972
94or378	unknown	-	-	-
100	Japan	Etoh 1985	"Takasaki-C"	1972
112	Japan	Etoh 1985	"Ishu-wase (Sakata)"	1980
124	Japan	Etoh 1985	"Kanchi-Howaito"	1980
129	Japan	Etoh 1985	"Iriomote"	1981
137	Peru	Etoh 1985	"Peru"	1981
144	Algeria	Etoh 1985	"Kabyle"	1981
180	Taiwan	-	"Taipei"	1983
199	Frunze	Etoh 1986	"Frunze-2"	1983
211	Moscow	Etoh 1986	"Moscow-5"	1983
222	Mexico	-	-	1983
225	Spain	-	"Spain-1"	1983
230	Japan	-	"Kawanabe-zairai"	1983
291	China	-	"Kunming"	1987
307	Greek	-	"Thessaloniki market-1"	1988
360	Japan	-	"Hiru"	1993
362	China	Hong and Etoh 1996	"Urumchi"	1994
369	Kazakhstan	Hong and Etoh 1996	"Almaty"	1994
397	China	Hong and Etoh 1996	"Kashgar"	1994
434	Spain	Etoh et al. 2001	"Spanish Gene Bank"	1996
445	Spain	Etoh et al. 2001	"Spanish Gene Bank"	1996
454	Spain	Etoh et al. 2001	"Spanish Gene Bank"	1996
462	Portugal	Etoh et al. 2001	"Portuguese Gene Bank"	1996
465	Portugal	-	"Braga Gene Bank"	1996
469	Portugal	-	"Braga Gene Bank"	1996
489	Egypt	-	"Egypt-2"	1999
490	Egypt	-	"Egypt-3"	1999
491	Jordan	-	"Jordan-1"	1999

Table 1. Continued.

Accession number	Collected country or site	Accession information	Remarks column	Year introduced to Kagoshima, Japan
493	Syria	-	"Syria-1"	1999
501	Japan	-	"Tarama"	1992
509	Thailad	-	"Chang Mai"	1992
521	China	-	"Sichuan"	1992
523	unknown	-	-	-
524	China	-	"Guizhou-D"	1992
534	China	-	"Guizhou"	1992
539	unknown	-	-	-
540	Japan	-	"Ishu-wase"	1992
542	Turkey	-	-	2001
552	Germany	Germany IPK collection All 130	-	2001
553	Germany	Germany IPK collection All 146	-	2001
554	Germany	Germany IPK collection All 780	-	2001
556	Germany	Germany IPK collection All 1035	-	2001
557	Germany	Germany IPK collection All 1038	-	2001
560	Germany	Germany IPK collection All 1473	-	2001
F17	Cenrtal Asia	-	-	-
F30	Cenrtal Asia	-	-	-
F31	Cenrtal Asia	-	-	-
F112	Cenrtal Asia	Hong et al. 2000a	-	1994
F115	Cenrtal Asia	Hong et al. 2000a	-	1994
F117	Cenrtal Asia	Hong et al. 2000a	-	1994
F138	Cenrtal Asia	Hong et al. 2000a	-	1994
F146	Cenrtal Asia	Hong et al. 2000a	-	1994
F147	Cenrtal Asia	Hong et al. 2000a	-	1994
F189	Cenrtal Asia	Hong et al. 2000a	-	1994
F215	Cenrtal Asia	Hong et al. 2000a	-	1994
F227	Cenrtal Asia	Hong et al. 2000a	-	1994
F424	Cenrtal Asia	Hong et al. 2000a	-	1994
F436	Cenrtal Asia	Hong et al. 2000a	-	1994
F1-200-23	Cenrtal Asia	Hong et al. 2000a	-	1994
F1-200-34	Cenrtal Asia	Hong et al. 2000a	-	1994
F1-200-92	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs401	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs404	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs405	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs407	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs410	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs407-410	Cenrtal Asia	unknown	-	-
Fs412	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs414	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs420	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs422	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs423	Cenrtal Asia	Hong et al. 2000a	-	1994
Fs424	Cenrtal Asia	Hong et al. 2000a	-	1994
Mai Dinh	Vietnam	-	"Mai Dinh"	-
IIT	India	-	-	-
Hagi	Japan	-	"Hagi"	-
Hirado	Japan	-	"Hirado"	-
Chang Mai small	Thailand	-	-	-
Chang Mai large	Thailand	-	-	-
Gatur	Turkey	-	-	-

Bolting traits of garlic collections

The bolting traits of garlic were examined in eight plants of the respective accessions and scored as previously described (Kamenetsky et al., 2005), with minor modifications. In brief, classification was decided as follows: Type A—bolters, producing mainly florets; Type B—bolters, producing mainly bulbils; Type C—incomplete bolters, scape covered with a false stem; and Type D—non-bolters, neither bolt nor develop flower buds. Before harvesting, in the middle of June, complete bolting accessions were classified as Type A or B, based on the produced flowers/bulbils ratio in the inflorescence. Developed inflorescences in each accessions were recorded in photo images. All garlic accessions were harvested at the end of June or the beginning of July and cured (completely dried of leaves and outer skins in a vented greenhouse). After that, their leaves, leaf sheaths, long scapes, and roots were removed. In these processes, accessions could be classified as Type C or D because they did not develop long scapes and inflorescences.

Extraction and determination of AlCSO (S-allyl-L-cysteine sulfoxide)

After curing, all accessions were stored in a vented greenhouse under ambient conditions from July to the middle of August. From the end of August to September, bulbs were used for chemical analysis. Extraction and determined AlCSO were used according to the method of Yaguchi et al., (2009), with minor modifications. All cloves were picked from at least three healthy bulbs in one accessions. Some cloves (A gram, A = 10 to 20) were microwaved for 2 min until the tissues were completely denatured of the alliinase enzyme. The tissues were weighed to calculate the amount of evaporated water (x g) and were homogenized with (A + x) g Mili-Q water for 10 min. The

homogenate was centrifuged at 4,000 × g for 10 min at room temperature, and the centrifuged sediments were removed. Every extract was stored at -20℃ until analysis. One mL of supernatant was again centrifuged at 15,000 × g for 1 min and was filtered by being passed through a 0.45-μm syringe-type filter (DISMIC-25cs; Advantec, Tokyo, Japan). A 25-μL filtered sample was injected into a high performance liquid chromatography (HPLC) system and quantified. The HPLC system included a pump, a degasser, a column oven, a diode array detector set to 220 nm, a data collection system (EZChrom EliteTM, Hitachi High-Technologies Corporation, Tokyo, Japan), and an AQUASIL SS-1251-120 column (4.6 mm i.d. × 250 mm long, Senshu Scientific Co., Ltd., Japan). The solvent was 0.005% trifluoroacetic acid and flowed for 15 min at a flow rate of 0.6 mL/min. Standard compounds were synthesized at Somatech Center (House Foods Corporation, Japan). The AlCSO standard was dissolved in Mili-Q water and analyzed as an external standard.

Phenolic content extraction and determination

All cloves were picked from at least three healthy bulbs in one accessions. All cloves of each garlic accessions had their outer skins removed and were cut into small pieces and mixed thoroughly. After that, 2 g of the clove tissue was extracted by 70% hot ethanol according to the method of Hang et al., (2004). Total phenolic content was determined using the method of Folin and Denis (1915), with minor modifications. Briefly, all extracts were diluted five times with distilled water. One mL of 1N phenol reagent (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was mixed with 1 mL of diluted extract. After 3 min, 1 mL of a 10% sodium carbonate aqueous solution was added, and the mixture was incubated for 60 min at room temperature. The absorbance

was measured at 530 nm on a U-2001 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan). Quantification of the phenolic content was based on a catechol calibration curve. All extracted samples were measured three times for each accessions.

Statistical analysis

All obtained chemical content data were used for a one-way analysis of variance (ANOVA), a Tukey's test, and a *t* test using SPSS 11.5 software with advanced models (SPSS Japan Inc., Tokyo, Japan).

Results and Discussion

Evaluation of garlic accessions for bolting traits

After harvesting and curing, bolting traits were evaluated in the mature garlic. There were variations in scape elongation or flower bud differentiations (Fig. 3). In all, 103 garlic accessions were examined, showing 44 bolters that produced mainly flowers (Type A), 35 bolters that produced mainly bulbils (Type B), 16 incomplete bolters (Type C), and 8 non-bolters (Type D). Their appearance frequencies were different among the geographical areas (Fig. 4). accessions collected from Central Asia and the Northern Mediterranean, high-latitude areas (approximately 40 °N and higher), produced florets and bulbils (less than 5 mm in size) in the inflorescence and were classified in Type A. These accessions would be the primitive style of garlic, due to their distance from the main center of diversity. Type A accessions in the Northern Mediterranean (accession

Nos. 434 and 445 from Spain; No. 469 from Portugal; and Nos. 552, 553, 554, 556, and 557 from Germany) were obtained from the gene bank, but no detailed information about their collected sites was available. Hong et al. (2000b) reported that Iberian garlic in Europe produced bulbils and malformed flowers in Kagoshima, the southern part of Japan. They suggested that this fact is evolutional evidence that reproductive organs turned into vegetative organs. Accessions collected from other areas (below 30 °N and in South America) produced only bulbils (more than 5 mm in size) or incomplete bolting and were classified in Type B or C. Type D (non-bolters) was seen in Japanese and European accessions (accession No. 56 from Japan; Nos. 144 and 491 from the Southeast Mediterranean; Nos. 225, 454, 462, and 465 from the Northern Mediterranean; and No. 523 from an unknown source). Accession No. 56 is from Japan, but its name is “California Early”. Therefore, this accession was probably derived from America. Etoh (1985) suggests that accessions collected from the areas of harsh cold winters with heavy snow, such as Europe, America, and northern Japan, have evolved to be non-bolting. In addition, he stated that some European accessions might have acquired bolting resistance due to farmers’ efforts to avoid a decrease in bulb yield. Therefore, garlic seems to have been strongly affected by selective pressure or various environmental conditions when it was introduced to the different regions.

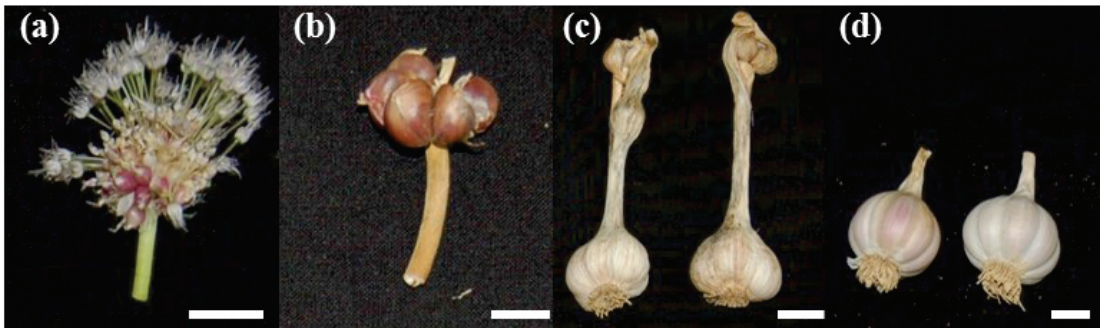

Fig. 3 Bolting traits of garlic in this study. **a** Type A=complete bolter (producing mainly flowers). Accession No. F147 from Central Asia; **b** Type B=complete bolter (producing mainly bulbils). Accession No.45 from Taiwan; **c** Type C=incomplete bolter. Accession No.6 from Japan; and **d** Type D=non-bolter. Accession No. 454 from Spain. Scale bar=15 mm.

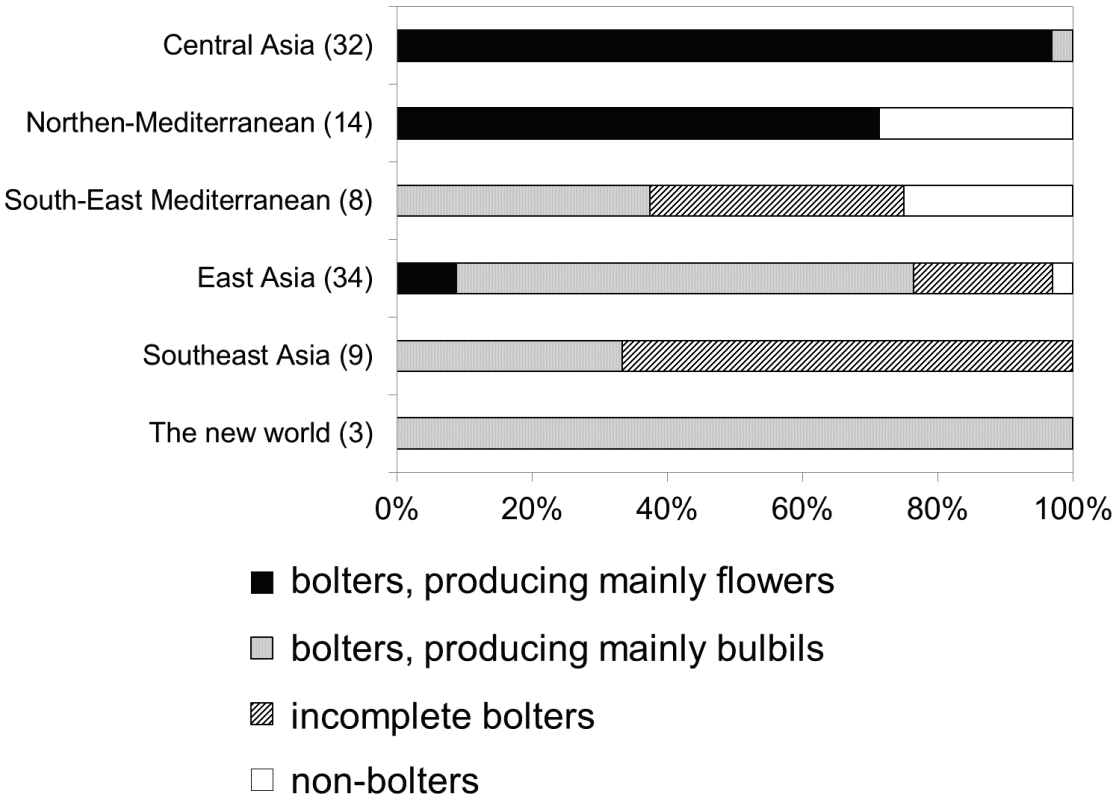

Fig. 4 Ratio of the different bolting types in 103 garlic accessions at the collected areas in 2009. Classification was carried out based on the inflorescence morphology shown in Fig. 3. Numbers in parentheses represent the number of accessions.

Determination of AlCSO (S-allyl-L-cysteine sulfoxide) and phenolic content in garlic collections

Based on the retention time and areas of the external standard, the AlCSO content of all garlic accessions was determined. AlCSO content varied from 2.33 (accession No. Fs424) to 10.26 (accession No. F146) mg/g FW and averaged 5.23 mg/g FW. Among samples from all of the collection areas, those from the Northern Mediterranean region had the highest AlCSO content (6.20 ± 0.23 mg/g FW) (Table 2). The ANOVA did not show the significance of the geographical sites. However, the coefficients of variation (CV) showed high levels in each collection region.

The phenolic content also varied greatly among all garlic bulb samples, from 52.29 (accession Hirado from Japan) to 137.52 (accession No. Fs420 from Central Asia) mg/100 g FW, and the average was 85.38 mg/100 g FW. Among the collection sites, Southeast Asia and the Southeast Mediterranean had high content (94.24 ± 6.40 and 90.86 ± 5.78 mg/100 g FW, respectively) (Table 2). However, garlic accessions from the New World had lower phenolic content. The phenolic content also showed a high CV, the same as the AlCSO content. Fig. 5 shows the variation of both chemical contents of garlic accessions among the geographical regions. There was a tendency for high AlCSO content in garlic to increase in high- and low-latitude areas (approximately 45 °N–40° N and 20 °N–15 °N, respectively). However, high-latitude areas tended to have low phenolic content. From these results, it seems that the production of both chemicals in garlic has been influenced by geographical distribution.

Table 2. Comparison of AlCSO and phenolic content of garlic accessions collected from various areas. Means followed by the same letter do not differ at 5% significance.

Collected areas	Number of Accessions	AlCSO (mg/g FW) Mean ± SE	CV(%)	phenolic contents (mg/100 g FW) Mean ± SE	CV(%)
Central Asia	32	5.47 ± 0.37 a	37.90	84.76 ± 3.86 a	15.20
Northen-Mediterranean	14	6.20 ± 0.23 a	13.67	76.61 ± 3.75 a	23.37
South-East Mediterranean	8	4.23 ± 0.29 a	19.46	94.24 ± 6.40 a	17.98
East Asia	34	4.81 ± 0.24 a	28.59	88.14 ± 2.80 a	20.26
Southeast Asia	9	5.91 ± 0.38 a	19.26	90.86 ± 5.78 a	18.18
The new world	3	4.70 ± 0.33 a	12.10	63.38 ± 2.63 b	6.55

[a]Means followed by the same letter do not differ at 5% significance

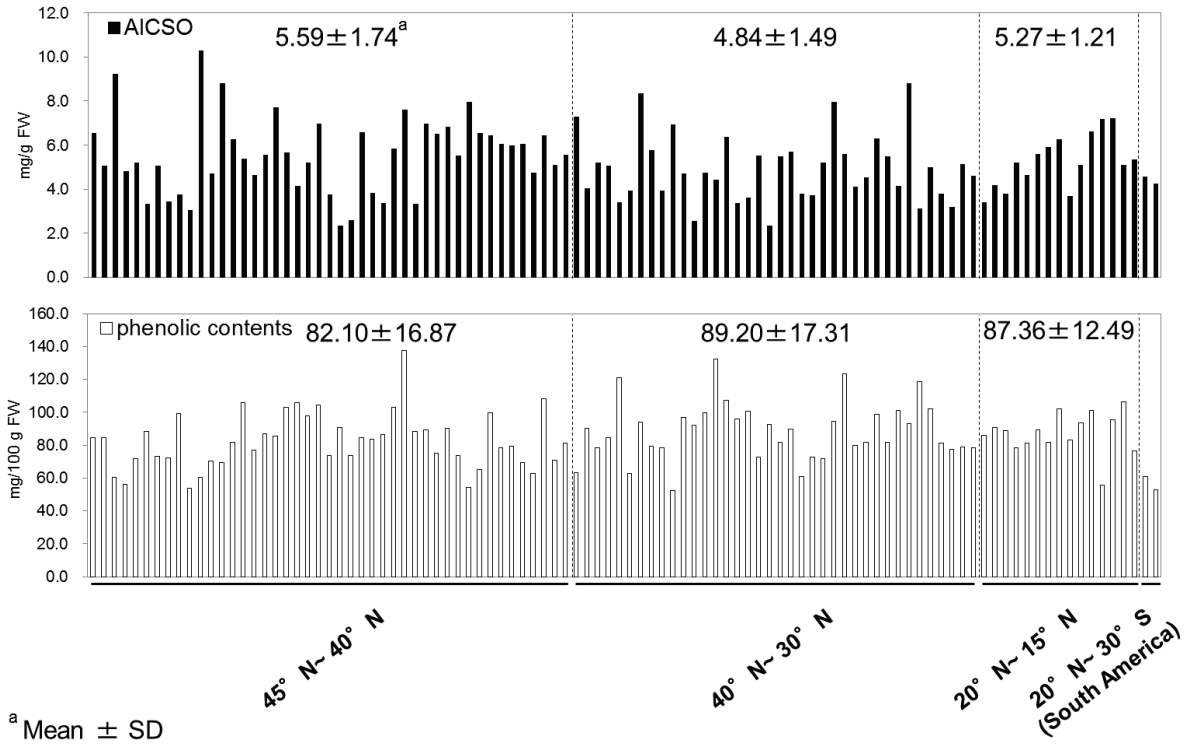

Fig. 5 Comparison of the AlCSO and phenolic content of garlic accessions among the geographical regions.

Relationship between bolting traits and chemical compound content

As mentioned above, the garlic accessions used in this study could be divided into four types according to their bolting traits. A comparison of these different types was carried out based on chemical composition data. Fig. 6 shows a comparison of both chemical contents among types. The AlCSO content was significantly different between Type B and D. Type D had the highest phenolic content, followed by Type C, Type B, and Type A. The content within types showed no significant difference by Tukey's test, but from Fig. 5, there would be high variability with notable differences between the accessions within each type. There was a tendency for the chemical components of garlic accessions to have differences among the types. Table 3 shows a comparison of the mean values of the chemical content between bolting types using the independent samples *t* test. The AlCSO content was significantly higher ($p < 0.05$) in Type A than in Type B. Moreover, a comparison of both chemicals' content was carried out in regions of various latitudes with a bolting type (Table 4). The high-latitude region (the Northern Mediterranean and Central Asia) consisted mainly of Types A and D. The AlCSO content in type A derived from this region was higher (5.54 ± 0.28 mg/g FW) than that derived from regions with a latitude of 30 °N–40 °N (4.75 ± 0.36 mg/g FW). However, the phenolic content was low in all types (74.72–82.20 mg/100 g FW). In regions where the latitude was 30 °N–40 °N (Japan, China, and the Southeast Mediterranean), all bolting types were present. In this region, the production levels of AlCSO and the phenolic content were almost the same among types except for Type D. The region with a latitude of 15 °N–30 °N (Southeast Asia and the small Japanese islands) contained Types B and C. In this region, Type C had high levels of both chemicals. In all geographical regions, Type D had high levels of both chemicals. Garlic accessions from

Peru and Chili, in the New World, showed much lower amounts of phenolic content than those from other regions (60.90 and 52.75 mg/100 g FW, respectively).

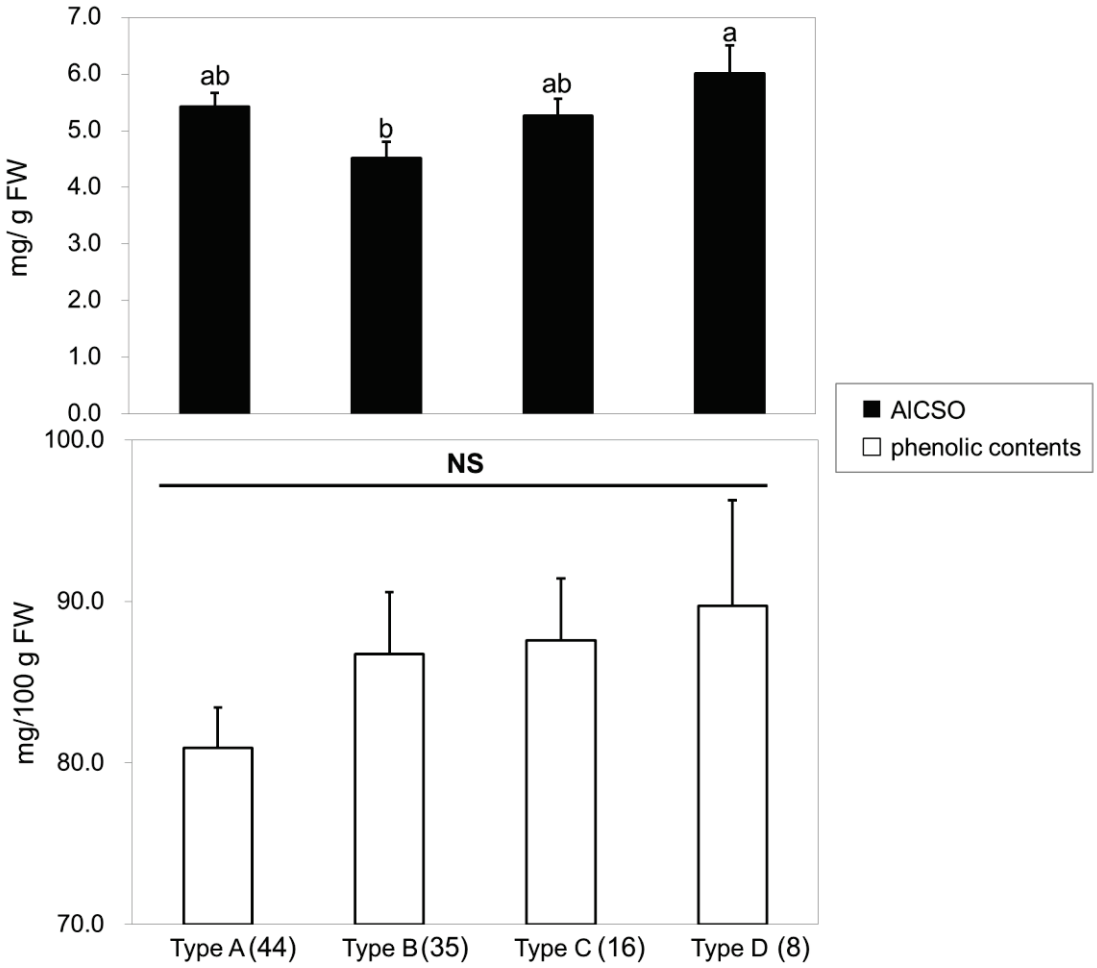

Fig. 6 Comparison of the AlCSO and phenolic content among the bolting types. Different letters significant difference and NS means no significance by Tukey's test ($p < 0.05$). Numbers in parentheses and error bars represent the number of clones and standard error, respectively.

Table 3. Independent samples *t* test for the comparison of the chemical contents between bolters.

Type	Number of accessions	AlCSO (mg/ g FW)	phenolic contents (mg/100 g FW)
		Mean ± SE	Mean ± SE
Bolters, producing mainly flowers	44	5.50 ± 0.27	79.18 ± 3.09
Bolters, producing mainly bulbils	35	4.66 ± 0.25	84.98 ± 3.39
t test		*	*ns*

* significant at $p < 0.05$, *ns* not significant

Table 4. Comparison of AlCSO and phenolic content based on the bolting type in respective geographical regions.

Geographical region	Collected area	Bolting type	Number of accessions	AlCSO (mg/ g FW)	phenolic contents (mg/100 g FW)
				Mean ± SE	Mean ± SE
40°N-45°N	Northen-Mediterranean, Central Asia	A	41	5.54 ± 0.28	82.20 ± 2.60
		B	1	3.12	74.72
		C	0	-	-
		D	4	6.09 ± 0.48	80.99 ± 9.83
		mean	46	5.55 ± 0.26	81.88 ± 2.49
30°N-40°N	Japan, China, South-East Mediterranean	A	3	4.75 ± 0.36	84.45 ± 3.37
		B	22	4.80 ± 0.36	90.47 ± 4.27
		C	9	4.74 ± 0.32	86.52 ± 5.07
		D	3	5.56 ± 1.24	92.64 ± 6.12
		mean	37	4.84 ± 0.25	89.20 ± 2.85
15°N-30°N	Southeast Asia, Japan (Island), Mexico	A	0	-	-
		B	8	4.71 ± 0.39	85.98 ± 2.89
		C	7	5.91 ± 0.40	88.93 ± 6.33
		D	0	-	-
		mean	15	5.27 ± 0.31	87.36 ± 3.22
The new world	Peru, Chili	A	0	-	-
		B	2	4.38	56.82
		C	0	-	-
		D	0	-	-
		mean	2	4.38	56.82

A:Bolters, producing mainly flowers B:Bolters, producing mainly bulbils C:Incomplete bolters D:Non-bolters

Although these garlic accessions were introduced in Japan and managed vegetatively for about 20 years in local climatic conditions, the examined garlic accessions showed remarkable biomorphological variations. As a whole, the AlCSO content in this study showed a tendency toward lower content than in previous reports (Yoo and Pike 1998). This might be due to the growing conditions. The Mediterranean climate is the best condition for garlic growing (Etoh 1985). The climate of the south area of Japan (high temperature and humidity in summer) affects garlic quality, and cysteine sulfoxide content also decreases. In addition, our accessions from Central Asia produced florets but did not show their fertility. Long vegetative propagation, especially in garlic, resulted in widespread infection by viruses and causes yield reductions or stunted plant development (Conci et al. 2002). This fact suggests that virus infection affects the formation level of bulbs or bulbils or seed fertility.

Etoh and Simon (2002) stated that the primitive forms of garlic originally produced umbels with mixed populations of flowers and topsets. Table 3 shows that there was a significant difference in the AlCSO content between complete bolting types. This means that as the distance from the high-latitude areas increases, garlic would be likely to produce bulbils in the inflorescence with lower AlCSO content and higher phenolic content. Etoh (1985) assumed that garlic might evolve from complete bolting to incomplete bolting or non-bolting accessions just after losing the capability of differentiating flower buds. In other *Allium* species, Vu et al. (2013) studied the biochemical diversity analysis of shallot germplasms in the Southeast areas and reported that the genetic variations in different regions would be derived from the adaptability of the plant to its local conditions during its cultivation history. It is highly probable that garlic was also selected naturally or artificially to make it adapt to environmental

conditions in various regions. Thousands of years of cultivation and selection by humans may have resulted in the evolution of garlic from sexual to asexual propagation. The production levels of chemical compositions in the bulbs may also have been affected. As a result, garlic seems to have obtained the environmental adaptability to survive unfavorable climatic conditions.

Chapter 3: QUANTITATIVE AND QUALITATIVE VARIATIONS OF SAPONIN PRODUCTION IN GENETIC RESOURCES OF GARLIC (*ALLIUM SATIVUM* L.) COLLECTED WORLDWIDE

Introduction

Garlic (*Allium sativum* L.) is one of the oldest plants, and it has been used as food and for medical applications since ancient times; its cultivation history dates to 3000 years BC (Figliuolo et al. 2001). The works of Vavilov (1951) and Kazakova (1971) indicated that garlic was originally from Central Asia; Vavilov (1951) proposed the Mediterranean area as a secondary center of origin. From this region, garlic has widely spread northwest (Europe) and southeast (Asia). It is strongly supported that *A. longicuspis* is an ancestral species. On the other hand, *Allium ampeloprasum* and *Allium tuncelianum* have been considered to be very close relatives to garlic. One trial investigated the phylogenetic relationship between garlic and these species (Ipek et al. 2008).

Allium plants are known for producing steroid saponins as well as organosulfur compounds (Lanzotti 2006). Saponins were reported as important secondary metabolites in *Allium* species involved in resistance to disease (Lanzotti 2005). These compounds are generally classified into two groups, triterpenoid saponins and steroid saponins, based on the molecular structure of aglycone. Steroid saponins are further divided into furostanol saponins and spirostanol saponins. Many steroid saponins have been reported in plants and animals, especially in the *Ailiaceae* family, which includes

garlic (Rivlin et al. 2006). In garlic, many kinds of saponins and sapogenins have been identified and their activities reported. However, few reports have evaluated the production level of saponins in garlic clones collected worldwide, including the primary and secondary centers of origin.

Garlic in various regions has accumulated mutations in order to adapt to different climatic conditions encountered in the expansion of garlic cultivation. It is expected that these mutations in garlic affect the chemical production levels of organic organosulfur, phenolic, and sugar compounds, as well as steroid saponins. In this chapter, we evaluate the quantitative and qualitative production levels of saponin compound content in order to clarify the production and variation levels.

Materials and Methods

Plant materials

Bulbs of 102 garlic accessions from Asia (24 accessions from Japan, six from China, nine from a tropical-subtropical area, and 29 from Central Asia), Europe (17 accessions from the Northern Mediterranean and 10 from the Southeastern Mediterranean), the New World (four accessions), and unknown (three accessions), as well as four *Allium ampeloprasum* (Great-headed garlic) accessions, and *Allium tuncelianum* have been collected from around the world since the 1970s and were managed in Yamaguchi University, Japan (34.14◦N, 131.47◦E). In short, a total 107 accessions, including five related *Allium* species, were used in this study (Table 5.). These bulbs were obtained from each country from local markets or national institutions. Some collected clones

have detailed information as reported by Etoh (1985; 1986), Hong et al. (2000), and Etoh et al. (2001). These bulbs were stored at 4°C in dark conditions during the summer. These accessions were planted in an experimental field at Yamaguchi University at the end of October 2011. A compound fertilizer was applied before planting with the method of Shigyo et al. (1997). The basal dressing contained three major nutrients in the following amounts: 100 N (as ammonium sulfate), 120 P (as calcium superphosphate), and 100 K (as potassium chloride) kg/ha. During the growing season, eight cloves of a uniform size were randomly selected from bulbs of each accession and were grown in rows 10 cm apart and in columns 20 cm apart. After harvest, the developed roots in each accession were washed to remove soils. Then, all accessions were dried in a vented greenhouse for a month to obtain dry roots.

Table 5. Garlic accessions and its related species used in this chapter.

No.	Managing number or name	Collected country or site	Accession information	Remarks column	Year introduced to Japan	Saponin contents Mean ± SE	saponin spot 1	2	3[a]	4	5[b]	6	7	8	9	10	11	12	13	14	15	16	17	18	Clade
1	5	Japan	Etoh 1985	‘‘Howaito-Roppen’’	1972	2.69 ± 0.06	+	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	1
2	6	Japan	Etoh 1985	‘‘Niigata-Sado’’	1972	1.02 ± 0.13	−	+	+	−	−	+	−	+	−	−	+	−	−	+	+	+	+	−	4
3	8	Japan	Etoh 1985	‘‘Ibaraki’’	1972	7.69 ± 0.06	+	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	1
4	14	Japan	Etoh 1985	‘‘Chiba-B’’	1972	11.60 ± 0.37	+	+	+	+	−	+	−	+	−	−	−	−	−	−	+	+	+	−	1
5	15	Japan	Etoh 1985	‘‘Hamamatsu’’	1972	10.78 ± 0.30	+	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	1
6	32	Japan	Etoh 1985	‘‘Iki-No. 1’’	1972	16.99 ± 0.89	+	+	+	+	−	+	−	+	−	+	+	−	−	−	+	+	+	−	2
7	37	Japan	Etoh 1985	‘‘Okute-B’’	1972	18.00 ± 0.70	+	+	+	+	−	+	−	+	+	+	+	−	−	−	+	+	+	−	2
8	39	Taiwan	-	‘‘Seira’’	1972	3.36 ± 0.14	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	6
9	40	Japan	Etoh 1985	‘‘Kokotsu’’	1972	12.81 ± 0.22	+	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	1
10	44	Taiwan	Etoh 1985	‘‘Taiwan-daikyu-pinku’’	1972	10.38 ± 0.65	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	6
11	45	Taiwan	Etoh 1985	‘‘Taiwan-shokyu-pinku’’	1972	13.49 ± 0.16	+	+	+	+	−	+	−	+	−	+	+	−	−	−	+	+	+	−	2
12	54	China	Etoh 1985	‘‘Fukushu (Foochow,China)’’	1972	8.05 ± 0.03	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
13	55	Egypt	Etoh 1985	‘‘Egypt’’	1972	4.27 ± 0.05	−	−	+	+	−	+	−	+	−	+	+	+	+	−	+	+	+	−	5
14	56	Japan	Etoh 1985	‘‘California Early’’	1972	6.77 ± 0.07	−	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
15	60	Chili	Etoh 1985	‘‘Chili’’	1972	16.32 ± 0.11	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
16	63	Japan	Etoh 1985	‘‘Saga-zairai’’	1972	7.53 ± 0.02	+	+	+	+	−	+	−	−	−	−	+	−	−	−	+	+	+	−	3
17	64	China	Etoh 1985	‘‘Shanhai-wase’’	1972	35.21 ± 1.50	+	+	+	+	−	+	−	+	−	+	+	+	−	−	+	+	+	−	2
18	65	Japan	Etoh 1985	‘‘Iki-shu’’	1972	7.82 ± 0.06	None[c]																		
19	67	Japan	Etoh 1985	‘‘Amami-A’’	1972	5.51 ± 0.05	+	+	+	+	−	+	−	+	+	−	+	+	−	−	+	+	+	−	1
20	68	Japan	Etoh 1985	‘‘Amami-B’’	1972	5.52 ± 0.07	−	−	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	5
21	69	Colombia	Etoh 1985	‘‘Colombia’’	1972	21.88 ± 1.05	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
22	75	Japan	Etoh 1985	‘‘Kushikino-wase’’	1972	7.64 ± 0.20	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
23	94or378	unknown	-	-	-	11.42 ± 0.19	+	+	+	+	−	+	−	+	−	+	+	−	−	−	+	+	+	−	2
24	100	Japan	Etoh 1985	‘‘Takasaki-C’’	1972	11.03 ± 0.19	+	+	+	+	−	+	−	+	+	−	+	−	−	−	+	+	+	−	1
25	112	Japan	Etoh 1985	‘‘Ishu-wase (Sakata)’’	1980	6.87 ± 0.04	−	−	+	+	−	+	−	+	+	+	+	+	+	−	+	+	+	−	5
26	124	Japan	Etoh 1985	‘‘Kanchi-Howaito’’	1980	23.96 ± 1.39	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
27	129	Japan	Etoh 1985	‘‘Iriomote’’	1981	9.25 ± 0.57	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
28	137	Peru	Etoh 1985	‘‘Peru’’	1981	9.48 ± 0.34	+	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	1
29	144	Algeria	Etoh 1985	‘‘Kabyle’’	1981	13.07 ± 0.45	+	+	+	+	−	+	−	−	−	−	+	−	−	−	+	+	+	−	3
30	180	Taiwan	-	‘‘Taipei’’	1983	19.38 ± 3.90	+	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	1
31	199	Frunze	Etoh 1986	‘‘Frunze-2’’	1983	6.37 ± 0.19	−	+	+	+	−	+	−	+	−	−	+	−	+	−	+	+	+	−	1
32	211	Moscow	Etoh 1986	‘‘Moscow-5’’	1983	8.06 ± 0.13	+	+	+	+	−	+	−	+	+	+	+	−	−	−	+	+	+	−	2
33	222	Mexico	-	-	1983	13.38 ± 0.16	+	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	1
34	225	Spain	-	‘‘Spain-1’’	1983	14.03 ± 0.54	+	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	1
35	230	Japan	-	‘‘Kawanabe-zairai’’	1983	8.71 ± 0.07	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
36	291	China	-	‘‘Kunming’’	1987	15.63 ± 0.52	+	+	+	−	−	+	−	+	−	+	+	+	−	−	+	+	+	−	2
37	307	Greek	-	‘‘Thessaloniki market-1’’	1988	17.55 ± 0.48	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
38	360	Japan	-	‘‘Hiru’’	1993	6.63 ± 0.21	−	+	+	+	−	+	−	+	+	+	−	−	+	−	+	+	+	−	5
39	362	China	Hong and Etoh 1996	‘‘Urumchi’’	1994	9.85 ± 0.16	+	+	+	+	−	+	−	+	−	−	+	−	+	−	+	+	+	−	1
40	369	Kazakhstan	Hong and Etoh 1996	‘‘Almaty’’	1994	44.79 ± 3.18	−	+	+	−	−	+	−	+	−	+	+	+	+	−	+	+	+	−	5

Table 5. Continued.

No.	Managing number or name	Collected country or site	Accession information	Remarks column	Year introduced to Japan	Saponin contents Mean ± SE	saponin spot 1	2	a 3	4	b 5	6	7	8	9	10	11	12	13	14	15	16	17	18	Clade
41	397	China	Hong and Etoh 1996	‘‘Kashgar’’	1994	14.38 ± 0.36	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
42	434	Spain	Etoh et al. 2001	‘‘Spanish Gene Bank’’	1996	12.22 ± 0.13	−	+	+	−	−	+	−	+	−	−	+	−	−	−	+	+	+	−	4
43	445	Spain	Etoh et al. 2001	‘‘Spanish Gene Bank’’	1996	6.54 ± 0.07	None																		
44	454	Spain	Etoh et al. 2001	‘‘Spanish Gene Bank’’	1996	4.84 ± 0.17	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
45	462	Portugal	Etoh et al. 2001	‘‘Portuguese Gene Bank’’	1996	9.96 ± 1.01	+	+	+	+	−	+	−	+	−	+	+	+	−	−	+	+	+	−	2
46	465	Portugal	-	‘‘Braga Gene Bank’’	1996	13.37 ± 0.69	+	+	+	+	−	+	−	+	+	−	+	+	−	−	+	+	+	−	1
47	469	Portugal	-	‘‘Braga Gene Bank’’	1996	14.06 ± 0.09	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
48	489	Egypt	-	‘‘Egypt-2’’	1999	32.18 ± 3.79	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
49	490	Egypt	-	‘‘Egypt-3’’	1999	6.14 ± 0.10	−	−	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	5
50	491	Jordan	-	‘‘Jordan-1’’	1999	13.40 ± 0.61	−	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
51	493	Syria	-	‘‘Syria-1’’	1999	6.01 ± 0.09	+	+	+	−	−	+	−	+	+	+	+	+	+	−	+	+	+	−	5
52	501	Japan	-	‘‘Tarama’’	1992	3.62 ± 0.22	−	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	4
53	509	Thailad	-	‘‘Chang Mai’’	1992	18.34 ± 3.90	−	+	+	+	−	+	−	+	+	−	−	−	−	−	−	+	+	−	6
54	523	unknown	-	-	-	19.10 ± 0.40	−	+	+	−	−	+	−	+	−	−	+	−	−	−	+	+	+	−	4
55	524	China	-	‘‘Guizhou-D’’	1992	8.15 ± 0.43	−	+	+	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	4
56	539	unknown	-	-	-	14.69 ± 0.09	+	+	+	+	−	+	−	+	−	+	+	−	−	−	−	+	+	−	2
57	540	Japan	-	‘‘Ishu-wase’’	1992	7.98 ± 0.04	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
58	542	Turkey	-	-	2001	3.38 ± 0.07	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
59	552	Germany	Germany IPK collection All 130	-	2001	2.97 ± 0.02	+	+	+	+	−	+	−	+	−	+	+	+	−	−	+	+	+	−	2
60	553	Germany	Germany IPK collection All 146	-	2001	12.72 ± 0.00	+	+	+	+	−	+	−	+	−	+	+	+	+	−	+	+	+	−	1
61	556	Germany	Germany IPK collection All 1035	-	2001	9.83 ± 0.06	+	+	+	+	−	+	−	+	−	+	+	+	+	−	+	+	+	−	1
62	557	Germany	Germany IPK collection All 1038	-	2001	18.25 ± 0.30	+	+	+	−	−	+	−	+	−	+	+	+	+	−	+	+	+	−	1
63	560	Germany	Germany IPK collection All 1473	-	2001	11.15 ± 0.20	+	+	+	+	−	+	−	+	−	−	+	−	−	+	+	+	+	−	1
64	F17	Cenrtal Asia	-	-	-	3.59 ± 0.14	+	+	+	−	−	+	−	+	+	−	+	+	+	−	+	+	+	−	5
65	F30	Cenrtal Asia	-	-	-	11.60 ± 0.21	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
66	F31	Cenrtal Asia	-	-	-	13.26 ± 0.03	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
67	F112	Cenrtal Asia	Hong et al. 2000	-	1994	1.12 ± 0.04	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
68	F115	Cenrtal Asia	Hong et al. 2000	-	1994	17.21 ± 0.53	−	+	+	−	−	+	−	+	−	−	+	+	−	−	+	+	+	−	4
69	F117	Cenrtal Asia	Hong et al. 2000	-	1994	5.32 ± 0.11	+	+	+	+	−	+	−	+	−	+	+	+	+	−	+	+	+	−	1
70	F138	Cenrtal Asia	Hong et al. 2000	-	1994	17.21 ± 0.26	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
71	F146	Cenrtal Asia	Hong et al. 2000	-	1994	7.06 ± 0.19	+	+	+	+	−	+	−	+	−	−	+	+	−	+	+	+	+	−	1
72	F147	Cenrtal Asia	Hong et al. 2000	-	1994	7.98 ± 0.02	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
73	F189	Cenrtal Asia	Hong et al. 2000	-	1994	6.86 ± 0.09	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
74	F215	Cenrtal Asia	Hong et al. 2000	-	1994	20.93 ± 1.62	+	+	+	+	−	+	−	+	−	+	+	+	−	−	+	+	+	−	2
75	F227	Cenrtal Asia	Hong et al. 2000	-	1994	7.34 ± 0.07	−	−	+	−	−	+	−	+	−	−	+	+	+	−	+	+	+	−	5
76	F424	Cenrtal Asia	Hong et al. 2000	-	1994	14.95 ± 0.21	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
77	F436	Cenrtal Asia	Hong et al. 2000	-	1994	7.87 ± 0.34	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
78	F1-200-23	Cenrtal Asia	Hong et al. 2000	-	1994	4.34 ± 0.20	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
79	F1-200-34	Cenrtal Asia	Hong et al. 2000	-	1994	17.53 ± 3.25	+	+	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	−	2
80	F1-200-92	Cenrtal Asia	Hong et al. 2000	-	1994	4.74 ± 0.18	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1

Table 5. Continued.

No.	Managing number or name	Collected country or site	Accession information	Remarks column	Year introduced to Japan	Saponin contents Mean ± SE	saponin spot 1	2	3 (a)	4	5 (b)	6	7	8	9	10	11	12	13	14	15	16	17	18	Clade
81	Fs405	Cenrtal Asia	Hong et al. 2000	-	1994	12.06 ± 0.66	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
82	Fs407	Cenrtal Asia	Hong et al. 2000	-	1994	9.60 ± 0.63	+	+	+	−	−	+	−	+	−	−	+	+	−	+	+	−	+	−	4
83	Fs410	Cenrtal Asia	Hong et al. 2000	-	1994	10.70 ± 0.34	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
84	Fs412	Cenrtal Asia	Hong et al. 2000	-	1994	10.41 ± 0.58	+	+	+	+	−	+	−	+	−	+	+	+	+	−	+	+	+	−	1
85	Fs414	Cenrtal Asia	Hong et al. 2000	-	1994	10.10 ± 0.26	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
86	Fs422	Cenrtal Asia	Hong et al. 2000	-	1994	12.73 ± 0.71	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	−	+	−	1
87	Fs423	Cenrtal Asia	Hong et al. 2000	-	1994	27.95 ± 2.71	+	+	+	−	−	+	−	−	−	−	+	−	−	−	+	+	+	−	3
88	Fs424	Cenrtal Asia	Hong et al. 2000	-	1994	11.07 ± 0.26	+	+	+	+	−	+	−	−	−	−	+	−	−	−	+	+	+	−	3
89	Mai Dinh	Vietnam	-	‘‘Mai Dinh’’	-	13.25 ± 2.44	+	+	+	+	−	+	−	+	−	+	+	+	−	−	+	−	+	−	2
90	IIT	India	-	-	-	7.12 ± 0.15	+	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
91	Hagi	Japan	-	‘‘Hagi’’	-	13.42 ± 1.21	−	+	+	+	−	+	−	+	−	−	+	+	−	−	+	+	+	−	1
92	Hirado	Japan	-	‘‘Hirado’’	-	3.54 ± 0.08	+	+	+	+	−	+	−	+	+	+	+	+	−	+	+	+	+	−	2
93	Egypt	Egypt		‘‘Aswan’’		17.00 ± 0.14	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	+	+	−	5
94	Chiang Mai small	Thailand	-	-	-	7.41 ± 0.75	+	+	+	−	−	+	−	+	−	+	+	+	−	−	+	+	+	−	2
95	Chiang Mai large	Thailand	-	-	-	15.17 ± 4.20	−	+	+	−	−	+	−	−	−	−	+	+	−	−	+	+	+	−	4
96	Gatur	Turkey	-	-	-	23.68 ± 4.25	+	+	+	+	−	+	−	+	+	+	+	+	−	+	+	+	+	−	2
97	Syria-1	Syria	-	-	-	8.31 ± 0.08	+	+	+	+	−	+	−	−	−	−	+	+	+	−	+	+	+	−	1
98	Syria-2	Syria	-	-	-	18.14 ± 0.42	−	−	+	+	−	+	−	+	−	−	−	+	+	−	+	+	+	−	5
99	Syria-3	Syria	-	-	-	11.20 ± 2.10	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
100	Syria-4	Syria	-	-	-	35.88 ± 2.31	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
101	Syria-5	Syria	-	-	-	47.71 ± 0.36	+	+	+	+	−	+	−	+	−	−	+	+	+	−	+	+	+	−	1
102	Kazakhstan	Kazakhstan	-	‘‘Chimkent’’	-	15.80 ± 0.80	−	+	+	+	−	+	−	+	+	+	+	+	+	−	+	+	+	−	5
103	224	-	Great-headed garlic	*A. ampeloprasum*	1994	7.08 ± 0.54	+	+	+	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	7
104	349	-	Great-headed garlic	*A. ampeloprasum*	1994	6.40 ± 0.07	+	+	+	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	7
105	461	-	Great-headed garlic	*A. ampeloprasum*	1996	6.02 ± 0.22	+	+	+	−	+	+	−	−	−	−	−	−	−	+	+	+	+	+	7
106	F310	-	Great-headed garlic	*A. ampeloprasum*	1988	6.15 ± 0.24	+	+	+	−	+	+	−	+	−	−	+	−	−	−	+	+	+	+	7
107	*A.tuncelianum*	Turkey	-	*A. tuncelianum*	-	8.34 ± 0.23	−	+	−	−	−	+	+	−	+	+	−	−	−	+	+	+	+	+	7

[a] β-chlorogenin spot

[b] agigenin spot

[c] Data not available

Extraction and quantifications of total saponins

The total saponin contents from the root portions of all accessions were extracted in accordance with the methods of Vu et al. (2013), with minor modifications. Briefly, the dry roots of each accession were bulked together and exhaustively extracted at room temperature with the solvent n-hexane to remove nonpolar compounds. The defatted materials were extracted with 80% methanol for 30 min of sonication and filtration twice. The extract was taken to dryness in a rotary evaporator with vacuum pump v-700 (Büchi, Rotavapor® R-3) under reduced pressure at 50ºC and then partitioned between butanol (BuOH) and H_2O (1:1). The BuOH layer was filtered and then concentrated under vacuum, causing a crude extraction of saponins. The total saponin content in the crude extract was determined spectrophotometrically at 473 nm, in accordance with the methods of Ebrahimzadeh and Niknam (1998), using 0.7% Vanillin-60% H_2SO_4 reagent. The absorbance was measured three times against the blank at 473 nm with a U-2000 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan). The major saponin compound in garlic, β-chlorogenin (Amagase 2006), was used as a standard to establish a calibration curve. The total saponin compound content was expressed as the β-chlorogenin equivalent per gram dry weight root (mg/g DW root).

Thin-layer chromatography (TLC) was used to detect the saponins in a butanolic fraction. After determining the saponin content in each accession, the concentration was adjusted to 20μg/μL by MeOH. The adjusted saponin was spotted on TLC, dried, and developed using a system of chloroform: methanol: water (12:6:1). Steroid saponins were visualized by spraying the TLC plates with *p*-anisaldehyde

reagents and heating them at 100°C for 10 min. In these saponin spots, spirostanol saponins were identified using Ehrlich reagent.

Data Analysis

All obtained data were subjected to Tukey's tests using SPSS 22.0 software (SPSS Japan Inc., Tokyo, Japan) to clarify the relationship between accessions' origin and their saponin contents. The spot patterns of saponins were studied using cluster analysis. Saponin spots were scored as present (+) or absent (-). The distance matrix was calculated using the PHYLIP (Phylogeny Inference Package) software package, Version 3.695 (Felsenstein 2002), and the phylogenic tree was constructed using the Mega program ver. 4.0 (Tamura et al. 2007).

Results

Quantity variation of saponins

Garlic clones showed saponin variations among all garlic samples, from 1.02 (accession '6' from Japan) to 47.71 (accession 'Syria-5' from the Southeastern Mediterranean) mg/g DW, and the average was 12.17 mg/g DW. However, saponin contents in *A. ampeloprasum* and *A. tuncelianum* were less than those in garlic (6.02–7.08 and 8.34 mg/g DW, respectively). Among the collection sites, China and the New World accessions had relatively higher contents (15.21 ± 6.40 and 15.58 ± 5.78 mg/g DW, respectively) than did those from other areas (Table 6.). ANOVA did not show the significance of the geographical sites. However, the coefficients of variation (CV) showed high levels in each collection region. Thus, it seems that the production of saponins in garlic has not been influenced by geographical distribution but depended on accessions.

Table 6. Comparison of saponin content of garlic accessions collected from various areas. Means followed by the same letter do not differ at 5% significance (Tukey's test).

Collected areas	Number of accessions	Total saponin (mg/gDW root) mean ± SE		CV (%)
Japan	24	14.05 ± 1.26	a	79.46
China	6	15.21 ± 1.48	a	67.72
Central Asia	29	11.54 ± 1.71	a	58.63
Tropical	9	11.45 ± 1.32	a	32.54
Northern Mediterranean	17	11.34 ± 1.26	a	34.41
South Eastern Mediterranean	10	14.61 ± 1.60	a	62.54
New World	4	15.58 ± 2.05	a	29.38
Unkown	3	15.07 ± 0.45	a	25.58

Quality variation of saponins in TLC spot profiling

Visualized saponin spots were numbered starting from the top and progressing to the bottom. β-chlorogenin and agigenin were also applied to identify saponins. A TLC saponin spot profiling showed clearly various pattern (Fig. 7). In garlic accessions, at least 15 spots were observed. In addition, by Ehrlich reagent staining, spot Nos. 13, 14, 15, 16, 17, and 18 were identified as spirostanol saponins. All garlic accessions had a β-chlorogenin spot (spot No. 3). Saponin spot Nos. 2, 3, 6, 8, 11, 15, 16, and 17 were observed in almost all garlic accessions. Thus, these spots were major saponins in garlic. However, some accessions did not produce these saponins. Moreover, in minor saponin spots, accessions showed high variation levels. Accessions from Central Asia and the Northern and Southeastern Mediterranean had a tendency to produce some specific saponins (spot No. 13 or 14). Accessions from these areas had a tendency to produce down parts of saponin spots, and other regions produced up parts of saponin spots. This result indicates that the garlic accessions have much diversity regarding the different kinds of saponins. On the other hand, the saponin profiles of the related species were different from those of garlic. *A. ampeloprasum* had an agigenin spot (spot No. 5) that garlic never possessed. In addition, this spot size differed among the same species. *A. tuncelianum* showed a unique spot profile. However, all species seem to share several spots (spot Nos. 15, 16, and 17).

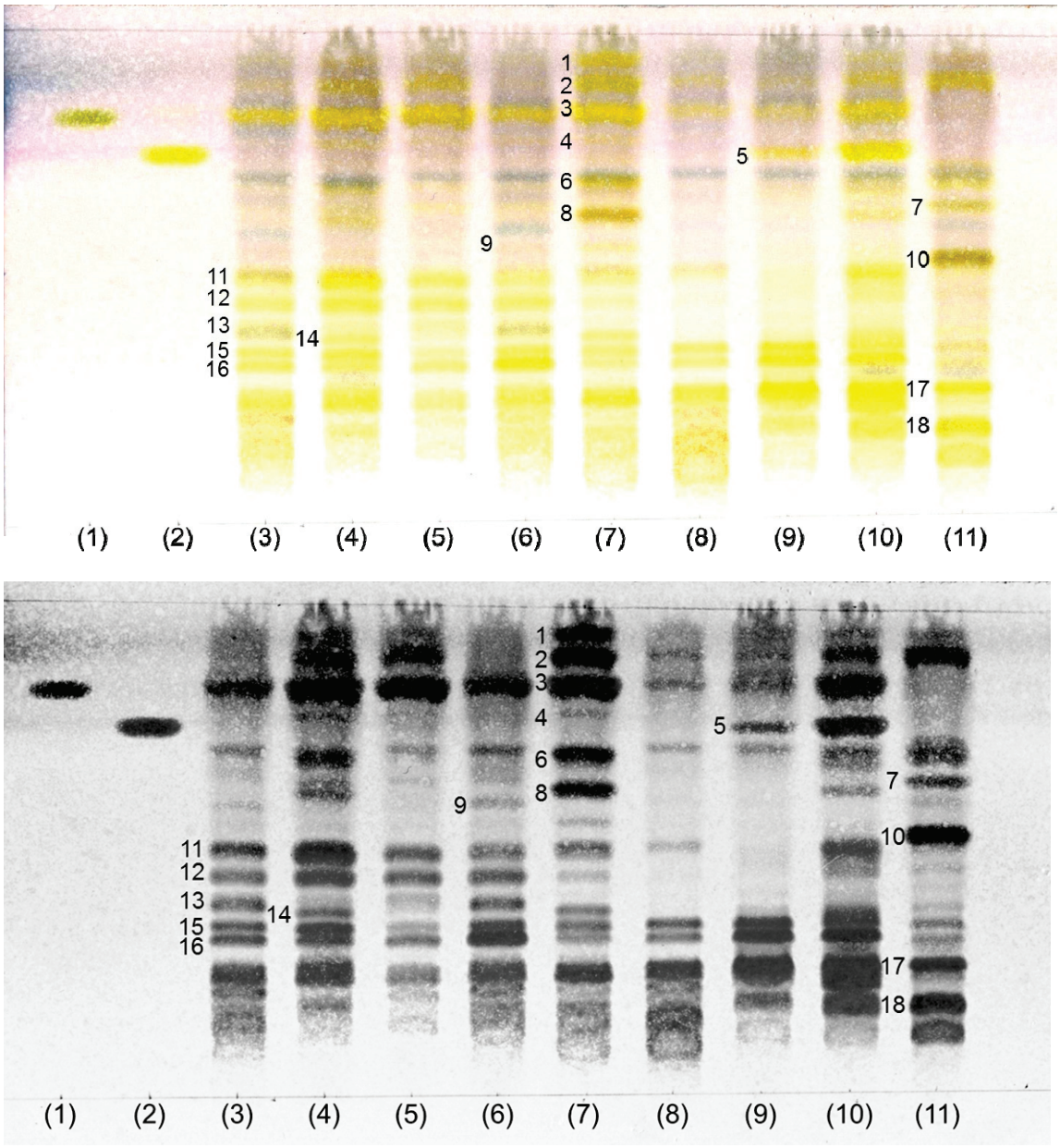

Fig. 7 TLC profiles for the qualitative analysis of saponins of garlic and its related species. **(1)** β-chlorogenin, **(2)** agigenin, **(3)** garlic accession 'Kazakhstan' from Central Asia, **(4)** garlic accession 'F138' from Central Asia, **(5)** garlic accession '490' from Southeast Mediterranean, **(6)** garlic accession '491' from Southeast Mediterranean, **(7)** garlic accession '60' from the New World, **(8)** garlic accession '63' from Japan, **(9)** *A. ampeloprasum* '349', **(10)** *A. ampeloprasum* 'F310', and **(11)** *A.tuncelianum.* Observed saponin spots were numbered starting from the top and progressing to the bottom.

Cluster analysis based on saponin spot profiling

Cluster analysis was performed to clarify the relationship between saponin spot profiles and collected regions. Based on saponin spot profiling, garlic and its related species were divided into seven clades (Fig. 8). *A. tuncelianum* was distinguished from other *Allium* species. Clade 7 consisted of four *A. ampeloprasum* accessions. Clade 1 consisted of 47 garlic accessions collected from various regions. These accessions had a slight saponin spot at No. 1 but did not have a spot at No. 14. Clade 2 consisted of 24 garlic accessions from various collected regions, and had the same result as Clade 1. However, these accessions had spot No. 10, which Clade 1 did not. Clade 3 consisted of four garlic accessions from Japan, Algeria, and Central Asia (accessions '63,' '144,' 'Fs423,' and 'Fs424'). These accessions did not produce saponin spot No. 8, which is regarded as a major saponin in garlic. Clade 4 consisted of six accessions from Japan, China, Central Asia, and Thailand (accessions '6,' '434,' '524,' 'F115,' 'Fs407,' and 'Chiang Mai large'). Clades 4, 5, and 6 had no saponin spot No. 1. Clade 5 accessions produced spot No. 13. Clade 6 consisted of three accessions from tropical-subtropical areas (accessions '39,' '44,' and '509'). These accessions produced only eight kinds of saponins (spot Nos. 2, 3, 4, 6, 8, 9, 16, and 17). These cluster analyses did not reflect the geographical origin directory. Each accession seems to produce its own specific saponins.

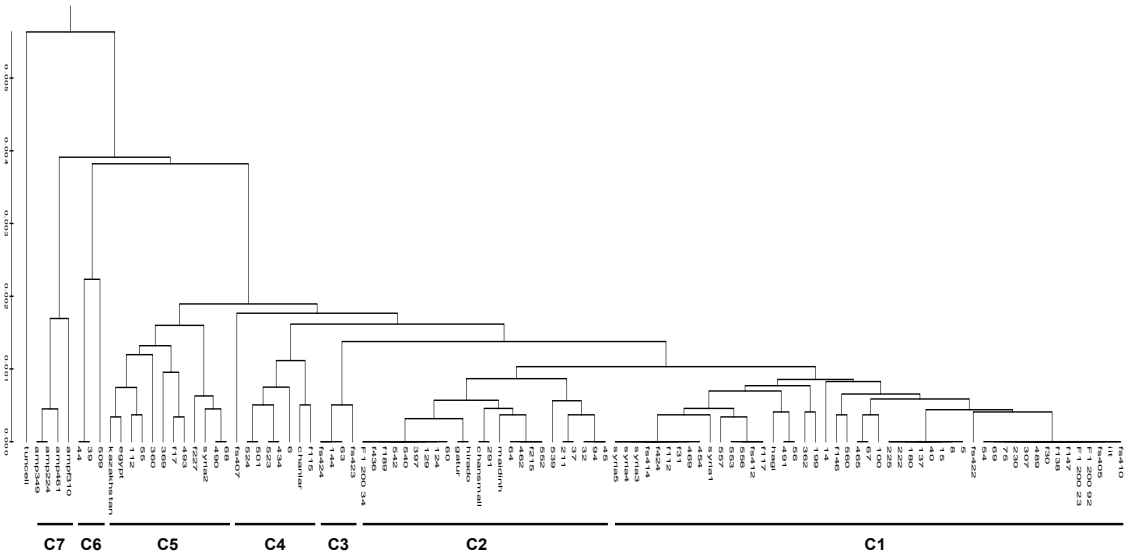

Fig. 8 Phylogenetic dendrogram of 105 accessions including five related *Allium* species based on saponin spot profiles (UPGMA)

Discussion

Although the garlic accessions used in this study grew in the same agroclimatic conditions, they showed variations in their amounts and types of steroid saponins. These results would indicate genetic variations. Saponin contents were not different among regions; however, they were varied among accessions. Additionally, with TLC assay, although we adjusted the same saponin concentrations of all accessions, the intensity of the saponin spots differed. Cluster analyses of saponins could be divided into seven clades. There was no significantly different saponin content among the clusters. The accessions in Clade 6 had less saponin spots than those in other clades. This was probably due to their history of cultivation. It is possible that these accessions might have been cultivated under abiotic stress. Meanwhile, Clade 5 accessions had four types of spirostanol saponins. It has been reported that spirostanol saponins are more active than furostanol saponins against the attacks of fungal pathogens (Matsuura 2001, Lanzotti et al. 2012). These accessions would have adapted against serious biotic stresses, such as soil pathogens. It would be possible for accessions from this cluster to have a high resistance to fungus.

Moreover, we could compare saponin contents and spot profiling in garlic and its related species, *Allium ampeloprasum* and *Allium tuncelianum*. Ipek et al. (2008) demonstrated this phylogenic relationship based on AFLP markers and nucleotide sequence analysis of the internal transcribed spacer region (ITS) and suggested that they are not immediate wild ancestral species of garlic. Garlic and its related species shared the same saponin spots that are never produced in other *Allium* species, such as the

onion (*Allium cepa*) or Japanese bunching onion (*Allium fistulosum*). This fact is interesting related to a discussion of their phylogenic relationship. Great-headed garlic resembles garlic but belongs to *Allium ampeloprasum* (Ariga et al. 2002). Additionally, it had already been reported that *Allium ampeloprasum* could produce agigenin, which is never produced in garlic (Morita et al. 1998). This compound could be used as a biomarker that can identify *A. sativum* and *A. ampeloprasum* (Amagase 2006). On the other hand, to the best of our knowledge, there has been no report regarding saponins in *Allium tuncelianum*. In this study, we obtained saponin profiling of this plant. Spot Nos. 2, 7, 10, and 18 were major saponin spots in this plant that were not observed clearly or were never seen in *A. sativum* and *A. ampeloprasum*. These compounds also could be used as biomarkers. In this study, we could only profile garlic accessions. The quantification of each saponin was uncertain. From TLC, it seems that the size of these spots differed among accessions. Further investigation about the production levels of each saponin and their relationships to disease tolerance is needed to confirm the antifungal activity of these plants.

Etoh (1985) supported a hypothesis that garlic might have evolved from fertility to sterility and from a complete-bolting type to a non-bolting type through an incomplete bolting type. In addition, garlic obtained specific adaptability to different agroclimatic regions (Figliuolo et al. 2001). It is likely that ancestral garlic populations would have had some fixed mutations. When they extended widely from their own growing field to different agroclimatic regions, only adaptable clones survived. It is possible that garlic has specifically adapted and spread to various agroclimatic conditions through the process of cultivation. Thus, it is highly probable that these agroclimatic changes have affected the production levels of chemical compositions in

garlic. It was assumed that garlic has adapted in various agroclimatic regions by producing unique saponin compounds over a long history of cultivation.

Chapter 4: DIVERSITY EVALUATION BASED ON THE MORPHOLOGICAL, PHYSIOLOGICAL, AND ISOZYME VARIATION IN GENETIC RESOURCES OF GARLIC (*ALLIUM SATIVUM* L.) COLLECTED WORLDWIDE

Introduction

Garlic is completely sterile; therefore, it has a long history of vegetative propagation. Garlic was surely cultivated in ancient Egypt; its cultivation history dates back to approximately 3000 BC, and it has been propagated, probably by bulbs or bulbils, since then (Figliuolo et al., 2001; Etoh 1985). Its presumed center of origin is considered to be the northwestern side of the Tien Shan Mountains, Central Asia, because fertile garlic, which is a primitive form, was found in this area (Etoh and Simon 2002). It is uncertain whether garlic became sterile after the beginning of its cultivation, but sterility in garlic is no doubt a consequence or a product of the species' evolution, including domestication (Etoh 1985).

For centuries, this plant has been propagated clonally, which has, perhaps, resulted in a bottleneck effect for genetic variation (Ma et al., 2009). However, cultivated garlic or clonal lineages exhibit remarkably wide morphological variation, such as in leaf number, bulb size and structure (such as arrangement, number, and size of the cloves), floral scape length, inflorescences (Pooler and Simon 1993, Keller 2002, Kamenetsky et al., 2005, Buso et al., 2008). Garlic has specific adaptations to different agroclimatic regions (Figliuolo et al., 2001; Mario et al., 2008). It is likely that, in

ancient times, ancestral garlic populations would have had some standing variations. When they extended widely from their own growing fields to different agroclimatic regions, only adaptable clones survived. Alternatively, after the start of domestication, different from the variation resulting from sexual reproduction, it is expected that the variation of domesticated garlic may exist due to mutations accumulated through the history of cultivation (Shaaf et al., 2014). Thus, it is possible that garlic has specifically adapted and spread to various agroclimatic environments through the process of domestication. As a result, garlic varied morphologically in various regions.

Characterization of garlic germplasm has been based largely on phenotypic characteristics. However, morphological characteristics can vary under different agroclimatic conditions (Jo et al., 2012). This situation causes complexity in the characterization of garlic clones (Mario et al., 2008). Many researchers have studied morphological traits and molecular markers such as isozymes and DNA to evaluate the diversity of garlic (Pooler and Simon 1993; Maass and Klaas 1995; Etoh et al., 2001; Zhao et al., 2011; Jo et al., 2012). Isozyme analysis has long been used to evaluate genetic diversity in animals, fungi, and higher plants (Micales and Bonde 1995). Lallemand et al., (1997) stated that the isozyme types of Central Asian clones were different from those of the Western world, and Asian clones are distinguished from those in other parts of the world in terms of isozyme types. Maass and Klaas (1995) categorized garlic species into four subspecies based on morphological and isozyme variations: the *longicuspis* group, including most garlic clones from Central Asia; the *subtropical* group, which developed in the climatic conditions of Southeast and East Asia; the *ophioscorodon* group, which is derived from Eastern Europe; and the *sativum* group, which is from the Mediterranean. However, reports evaluating morphological

characteristics and isozyme polymorphisms of garlic, including these subspecies, have been limited.

In this chapter, we investigate morphological and physiological traits and some isozymes of garlic accessions collected worldwide in order to evaluate garlic's diversity.

Materials and Methods

Plant materials

Bulbs of 107 garlic accessions collected from around the world since the 1970s have been managed at Yamaguchi University, Japan (34.14 °N, 131.47 °E). In addition, bulbs of 33 garlic accessions were managed at Saga University, Japan (33.24 °N, 130.29 °E) until 2012, when management of these collections was taken over by Yamaguchi University. The following eight groups were categorized based on their origins. 31 accessions from Honshu, Japan (Group A), 18 accessions from islands in Western Japan (Group B), 10 accessions from China, 16 accessions from Southeast Asia, 29 accessions from Central Asia, 13 accessions from the Northern Mediterranean, 14 accessions from the Southeastern Mediterranean, and six accessions from The New World. Thus, a total of 140 accessions (including three from unknown origins) were used in this chapter (Table 7). These bulbs were obtained from local markets or national institutions in each country. Detailed information regarding some accessions was reported by Etoh (1985), Etoh (1986), Hong et al. (2000), and Etoh et al. (2001). These accessions have no clear information as to which subspecies they belong; however, they include four subspecies. These bulbs were stored at 4 °C in dark conditions in the summer.

The 140 garlic accessions were planted in an experimental field at Yamaguchi University at the end of October 2011 and 2012. A compound fertilizer was applied before planting. Total amounts of three major nutrients in a basal dressing were 100 N (as ammonium sulfate), 120 P (as calcium superphosphate), and 100 K (as potassium chloride) kg/ha. During the growing season, eight cloves of a uniform size per accession were randomly selected from the bulbs and were grown in rows 10 cm apart and in columns 20 cm apart.

Morphological and physiological observations

In 2011, morphological and physiological traits of the 107 accessions were examined for eight plants of each accession. Bolting types of the accessions were identified as follows: complete bolting—plant always bolts, its scape elongates high above the ground, and the inflorescence comes out of leaf sheath; incomplete bolting—plant produces a thin, short scape and bears only a few bulbils in its leaf sheath; non-bolting—plant neither bolts nor develops flower buds. The identification was carried out in accordance with the guidelines of Kamenetsky et al., (2005), with minor modifications. The bolting period was recorded as a physiological trait by counting the days until the accession's spathes differentiated. In incomplete-bolting accessions, the number of days to the development of bulbils in the leaf sheath was recorded. Just before harvest season, accessions that did not develop scapes and inflorescences could be identified as the non-bolting type. These accessions were removed from the survey of the bolting period. All accessions were harvested and cured (completely dried of umbels, scapes, leaves, leaf sheaths, and roots) in a vented greenhouse. About one month later, the scape length, number of bulbils per accession,

and bulb-related traits (bulb weight, bulb diameter, and number of cloves) per accession were recorded with mature plants. The number of bulbils per accession was determined by counting all bulbils removed from umbels divided by the number of umbels examined.

Isozyme analysis

In April and May, phenotypes of two enzymes, leucine aminopeptidase (LAP; E.C. 3.4.11.1) and phosphoglucoisomerase (PGI; E.C. 5.3.1.9), in the 140 garlic accessions were analyzed using polyacrylamide gel electrophoresis. The newly expanding leaves, approximately five cm long from tip to tip, were collected from all accessions to be analyzed for enzymes. Enzyme extraction, electrophoresis, and staining were carried out following the method of Shigyo et al., (1995 and 1996). Minor modifications were applied to the extraction buffer (Wendel 1983) for enzyme extraction. The polyacrylamide gel was composed of Tris-HCl running gel (7% acrylamide, pH 8.9) and Tris-HCl stacking gel (4.2% acrylamide, pH 8.9). The crude enzymes extracted from the leaves were loaded into the wells of gel at doses of 20 μL and 10 μL of each LAP and PGI sample, respectively. The number of alleles and allelic frequencies at each isozyme gene were evaluated for each group of accessions, defined by their origin

.

Data analysis

All obtained morphological (scape length, number of bulbils per accession, and bulb-related traits) and physiological (bolting period) data from the groups' accessions by their origins were subjected to various statistical tests. A principal component

analysis (PCA) was completed using SPSS 22.0 statistical software (SPSS Japan, Inc., Tokyo, Japan) to clarify the relationship between the morpho-physiological data and the collection site. In each group, we examined whether the allele frequencies were the same as the average of frequencies across all samples using a chi-square test. Genetic diversity can be measured by heterozygosity (Nei 1973). Thus, to evaluate genetic diversity at the isozyme loci, the observed (H_o) and expected (H_e) heterozygosity in each group were calculated. In addition, deviations from the Hardy–Weinberg equilibrium (HWE) in each locus were evaluated by a chi-square test. G_{st} values were calculated using GenAlEx ver. 6.5 software for Windows (Peakall and Smouse 2012) to show the level of geographical differentiation. MANOVA (multivariate analysis of variance) was carried out on the obtained data to clarify the relationships between morpho-physiological characteristics, isozyme genotypes, and the group of origin of the accessions.

Table 7. Garlic accessions used in this chapter.

No.	Collected country or site (Geographical region)	Managing number or name	Collected country or site	Accession information	Remarks column
1	Japan - Group A (30°N-40°N)	5	Japan	Etoh 1985	''Howaito-Roppen''
2		6	Japan	Etoh 1985	''Niigata-Sado''
3		8	Japan	Etoh 1985	''Ibaraki''
4		9	Japan	Etoh 1985	''Chiba-A''
5		15	Japan	Etoh 1985	''Hamamatsu''
6		16	Japan	Etoh 1985	''Wakayama-Roppen''
7		37	Japan	Etoh 1985	''Okute-B''
8		40	Japan	Etoh 1985	''Kokotsu''
9		56	Japan	Etoh 1985	''California Early''
10		63	Japan	Etoh 1985	''Saga-zairai''
11		75	Japan	Etoh 1985	''Kushikino-wase''
12		100	Japan	Etoh 1985	''Takasaki-C''
13		124	Japan	Etoh 1985	''Kanchi-Howaito''
14		230	Japan	-	''Kawanabe-zairai''
15		360	Japan	-	''Hiru''
16		Hagi	Japan	-	''Hagi''
17		Hirado	Japan	-	''Hirado''
18		Chugokukei ninniku	Japan	-	-
19		Chiba-shoukyu	Japan	Saga university, Japan	-
20		Enhei	Japan	Saga university, Japan	-
21		Enshuu-gokuwase	Japan	Saga university, Japan	-
22		Kagoshima	Japan	Saga university, Japan	-
23		Kashu-wase	Japan	Saga university, Japan	-
24		Kashu-okute	Japan	Saga university, Japan	-
25		Katish(touhiru)	Japan	Saga university, Japan	-
26		Kouchi-shoukyuu	Japan	Saga university, Japan	-
27		Nagano	Japan	Saga university, Japan	-
28		Saga-ooninnniku	Japan	Saga university, Japan	-
29		S62-tashirouetuke	Japan	Saga university, Japan	-
30		Setouchi	Japan	Saga university, Japan	-
31		Wase-ninniku	Japan	Saga university, Japan	-
32	Japan - Group B (20°N-30°N)	32	Japan	Etoh 1985	''Iki-No. 1''
33		65	Japan	Etoh 1985	''Iki-shu''
34		67	Japan	Etoh 1985	''Amami-A''
35		68	Japan	Etoh 1985	''Amami-B''
36		112	Japan	Etoh 1985	''Ishu-wase (Sakata)''
37		129	Japan	Etoh 1985	''Iriomote''
38		501	Japan	-	''Tarama''
39		540	Japan	-	
40		Okinawa(naha)	Japan	-	''Naha''
41		Okinawa(tamagusuku)	Japan	-	''Tamagusuku''
42		Okinoerabu	Japan	Saga university, Japan	-
43		Kikai(onodu)	Japan	Saga university, Japan	-
44		Kikai(oodama)	Japan	Saga university, Japan	-
45		Kikai(ikumi)	Japan	Saga university, Japan	-
46		Iki-ooninnniku	Japan	Saga university, Japan	-
47		Iki-wase	Japan	Saga university, Japan	-
48		Okinawa-nanbu	Japan	Saga university, Japan	-
49		Taishu-san	Japan	Saga university, Japan	-
50	China (30°N-40°N)	54	China	Etoh 1985	''Fukushu (Foochow,China)''
51		64	China	Etoh 1985	''Shanhai-wase''
52		291	China	-	''Kunming''
53		362	China	Hong and Etoh 1996	''Urumchi''
54		397	China	Hong and Etoh 1996	''Kashgar''
55		524	China	-	''Guizhou-D''
56		534	China	-	''Guizhou''
57		Kankousan	China	Saga university, Japan	-
58		Hongkong-wase	China	Saga university, Japan	-
59		Shang-hai	China	Saga university, Japan	-
60	Southeast Asia (15°N-20°N)	39	Taiwan	-	''Seira''
61		44	Taiwan	Etoh 1985	''Taiwan-daikyu-pinku''
62		45	Taiwan	Etoh 1985	''Taiwan-shokyu-pinku''
63		180	Taiwan	-	''Taipei''
64		509	Thailad	-	''Chang Mai''
65		Mai Dinh	Vietnam	-	''Mai Dinh''
66		IIT	India	-	-
67		Chang Mai small	Thailand	-	-
68		Chang Mai large	Thailand	-	-
69		67-4	Thailand	Saga university, Japan	-
70		151-1	Thailand	Saga university, Japan	-
71		73-4	Thailand	Saga university, Japan	-
72		16-5	Thailand	Saga university, Japan	-

Table 7. Continued.

No.	Collected country or site (Geographical region)	Managing number or name	Collected country or site	Accession information	Remarks column
73		174-1	Thailand	Saga university, Japan	-
74		210-3	Thailand	Saga university, Japan	-
75		202-1	Thailand	Saga university, Japan	-
76	Central Asia (40°N-45°N)	199	Frunze	Etoh 1986	‘‘Frunze-2’’
77		211	Moscow	Etoh 1986	‘‘Moscow-5’’
78		369	Kazakhstan	Hong and Etoh 1996	‘‘Almaty’’
79		F17	Cenrtal Asia	-	-
80		F30	Cenrtal Asia	-	-
81		F31	Cenrtal Asia	-	-
82		F112	Cenrtal Asia	Hong et al. 2000	-
83		F115	Cenrtal Asia	Hong et al. 2000	-
84		F117	Cenrtal Asia	Hong et al. 2000	-
85		F138	Cenrtal Asia	Hong et al. 2000	-
86		F146	Cenrtal Asia	Hong et al. 2000	-
87		F147	Cenrtal Asia	Hong et al. 2000	-
88		F189	Cenrtal Asia	Hong et al. 2000	-
89		F215	Cenrtal Asia	Hong et al. 2000	-
90		F227	Cenrtal Asia	Hong et al. 2000	-
91		F424	Cenrtal Asia	Hong et al. 2000	-
92		F436	Cenrtal Asia	Hong et al. 2000	-
93		F1-200-23	Cenrtal Asia	Hong et al. 2000	-
94		F1-200-34	Cenrtal Asia	Hong et al. 2000	-
95		F1-200-92	Cenrtal Asia	Hong et al. 2000	-
96		Fs405	Cenrtal Asia	Hong et al. 2000	-
97		Fs407	Cenrtal Asia	Hong et al. 2000	-
98		Fs410	Cenrtal Asia	Hong et al. 2000	-
99		Fs412	Cenrtal Asia	Hong et al. 2000	-
100		Fs414	Cenrtal Asia	Hong et al. 2000	-
101		Fs422	Cenrtal Asia	Hong et al. 2000	-
102		Fs423	Cenrtal Asia	Hong et al. 2000	-
103		Fs424	Cenrtal Asia	Hong et al. 2000	-
104		Kazakhstan	Cenrtal Asia		‘‘Chimkent’’
105	Northern Mediterranean (40°N-45°N)	225	Spain	-	‘‘Spain-1’’
106		307	Greek	-	‘‘Thessaloniki market-1’’
107		434	Spain	Etoh et al. 2001	‘‘Spanish Gene Bank’’
108		445	Spain	Etoh et al. 2001	‘‘Spanish Gene Bank’’
109		454	Spain	Etoh et al. 2001	‘‘Spanish Gene Bank’’
110		462	Portugal	Etoh et al. 2001	‘‘Portuguese Gene Bank’’
111		465	Portugal	-	‘‘Braga Gene Bank’’
112		469	Portugal	-	‘‘Braga Gene Bank’’
113		552	Germany	Germany IPK collection All 130	-
114		553	Germany	Germany IPK collection All 146	-
115		556	Germany	Germany IPK collection All 1035	-
116		557	Germany	Germany IPK collection All 1038	-
117		560	Germany	Germany IPK collection All 1473	-
118	Southeastern Mediterranean (30°N-40°N)	55	Egypt	Etoh 1985	‘‘Egypt’’
119		144	Algeria	Etoh 1985	‘‘Kabyle’’
120		489	Egypt	-	‘‘Egypt-2’’
121		490	Egypt	-	‘‘Egypt-3’’
122		491	Jordan	-	‘‘Jordan-1’’
123		493	Syria	-	‘‘Syria-1’’
124		542	Turkey	-	-
125		Egypt	Egypt	-	‘‘Aswan’’
126		Syria-1	Syria	-	-
127		Syria-2	Syria	-	-
128		Syria-3	Syria	-	-
129		Syria-4	Syria	-	-
130		Syria-5	Syria	-	-
131		Gatur	Turkey	-	-
132	The New World (20°N-30°S)	60	Chili	Etoh 1985	‘‘Chili’’
133		69	Columbia	-	-
134		137	Peru	Etoh 1985	‘‘Peru’’
135		222	Mexico	-	-
136		Chili	chili	Saga university, Japan	-
137		Columbia	columbia	Saga university, Japan	-
138	Unknown	94or378	unknown	-	-
139		523	unknown	-	-
140		539	unknown	-	-

Results

Morphological and physiological variation

Garlic accessions showed various morphological variations when grown in the experimental field at Yamaguchi University. The number of cloves/plants varied among the accessions from two to 44. Almost all garlic accessions developed several cloves, ranging from two to 20 among the groups (Fig. 9A), except for the Southeastern Mediterranean group. Accessions from this group developed many cloves, ranging from seven to 44 (Fig. 9B). All accessions of the Central Asia group developed complete bolting. These accessions produced many more small bulbils (less than 5 mm in size) with florets than did those from other groups (Fig. 9C and D). Incomplete bolting was also observed in some accessions (Fig. 9E). These accessions were mainly seen in the Southeast Asia and Southeastern Mediterranean groups. Morphological and physiological traits were compared among groups of accessions classified by their origins (Table 8). The scape length and number of bulbils per plant in the Central Asia group were significantly greater than those in Japan Groups A and B and the Southeast Asia group; however, the China group had traits similar to those of Central Asia in scape length and the number of bulbils. In bulb weight, the Central Asia group, Japan Group A, and the Southeast Asia group showed similar values. Southeastern Mediterranean accessions produced significantly heavier bulbs (25.0 ± 3.7 g) than did those of other groups. While there was no significant difference in clove weight among the regional groups, the number of cloves in Southeastern Mediterranean accessions was greater than that of accessions of the other groups. Bolting periods in Southeast

Asia accessions were significantly shorter (179.2 ± 2.8 days) than those in other groups. Regarding bolting types, all Central Asia and China accessions bolted completely, while accessions from the other groups bolted incompletely or did not bolt. In particular, Southeastern Mediterranean and Southeast Asia accessions had high ratios of these bolting types.

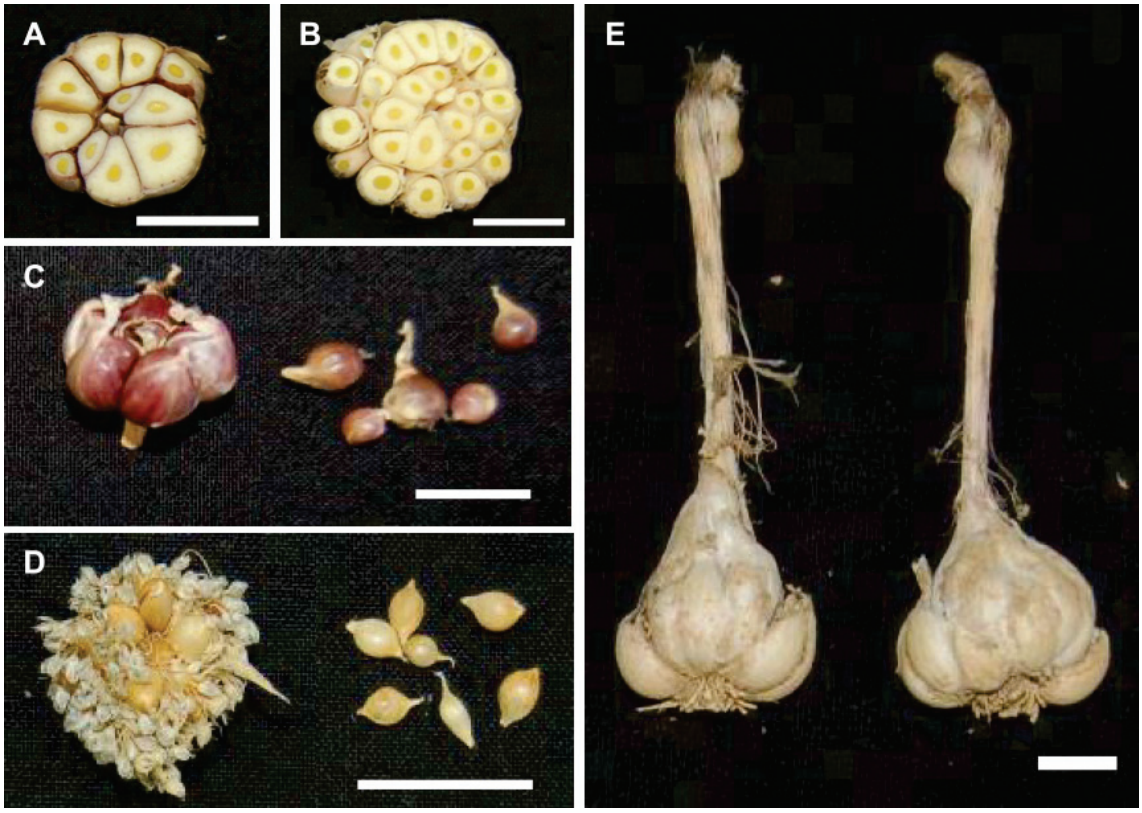

Fig. 9 Various morphological traits of garlic accessions observed in this chapter. Scale bars indicate 15mm. (A) Bulb structure and cloves in accession No. 230 (Japan). (B) Bulb structure and cloves in accession No. 490 (Egypt). (C) Produced bulbils in accession No. 45 (Taiwan). (D) Produced bulbils in accession No. F147 (Central Asia). (E) Incomplete bolting. Accession No. 39 (Taiwan).

Table 8. Comparison of morphological and physiological traits of garlic accesions collected from various areas. Means followed by the different letters differ at 5% significance by Tukey's test.

Groups		Scape length (cm)		Number of bulbils		Bulb weight (g)		Bulb diameter (cm)		Number of cloves		Clove weight (g)		Bolting period (day)		Bolting type		
		N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	N	Mean ± SE	CB[a]	IB	NB
Central Asia		27	81.7 ± 3.3 b	25	66.9 ± 5.8 b	27	9.6 ± 1.0 a	27	2.8 ± 0.1 ab	27	6.9 ± 0.3 a	27	1.4 ± 0.1 a	25	191.3 ± 1.3 b	25	0	0
Northen Mediterranean		12	52.4 ± 7.5 ab	9	57.5 ± 13.9 ab	11	12.6 ± 2.6 a	11	3.0 ± 0.3 a	9	7.4 ± 0.9 a	9	1.9 ± 0.2 a	7	189.9 ± 2.6 ab	10	0	2
Southeastern Mediterranean		7	63.5 ± 6.8 ab	12	34.2 ± 12.5 a	14	25.0 ± 3.7 b	14	4.4 ± 0.3 b	14	19.3 ± 3.7 b	14	1.7 ± 0.3 a	12	185.5 ± 1.4 ab	3	9	2
China		6	60.9 ± 10.2 ab	5	54.8 ± 14.9 b	6	14.4 ± 3.0 ab	6	3.4 ± 0.3 ab	6	7.8 ± 0.5 a	6	1.8 ± 0.3 a	6	181.3 ± 3.2 b	6	0	0
Japan	Group A	16	35.6 ± 4.2 a	14	10.9 ± 2.7 a	16	8.4 ± 1.1 a	16	2.8 ± 0.2 ab	16	6.2 ± 0.4 a	16	1.4 ± 0.2 a	14	181.9 ± 1.4 b	13	3	1
	Group B	8	39.7 ± 9.1 a	7	17.1 ± 3.7 a	8	11.8 ± 1.8 ab	8	3.3 ± 0.2 ab	8	9.0 ± 2.4 ab	8	1.9 ± 0.6 a	8	181.3 ± 1.1 b	5	3	0
Southeast Asia		6	40.0 ± 11.2 a	6	18.1 ± 8.4 a	9	9.1 ± 2.2 a	9	2.9 ± 0.3 ab	9	5.8 ± 1.1 a	9	1.7 ± 0.4 a	5	179.2 ± 2.8 a	2	4	3
The New World		2	83.3	2	19.8	3	15.8 ± 5.8 ab	3	3.7 ± 0.7 ab	3	6.5 ± 0.1 a	3	2.4 ± 0.9 a	2	188.5	2	0	1

[a] CB; Complete Bolting, IB; Incomplete Bolting, NB; Non-Bolting

Isozyme variation

Multiple loci in one enzyme system were numbered starting from the cathode and progressing to the anode (Fig. 10). These alleles were arranged alphabetically, starting with the slowest band. LAP and PGI putative genotypes at *Lap-1*, *Lap-2*, or *Pgi-1* showed clear polymorphism. *Lap-1* was detected in a single band (genotypes 'aa' and 'bb') and two bands (genotype 'ab') because LAP is a monomeric enzyme (Maass and Klaas 1995). In this study, the 'ab' band did not show clearly for technical reasons, such as non-optimal enzyme volume or polyacrylamide gel concentrations. *Lap-2* was detected clearly in several banding patterns (genotypes 'ac,' 'bb,' 'bc,' 'bd,' 'cc,' and 'cd'). In PGI, single or triple bands were detected. This result indicated the following: *Pgi* was composed of (1) a single gene or (2) two genes. In the former case, there are two bands of homodimers with heterodimers. In the latter, there are two *Pgi* genes that form an intergenic heterodimer. Some reports have indicated that in several plants, such as rice and tomatoes, a heterodimer of the products of the two isozyme genes is formed (Weeden et al., 1979; Tanksley et al., 1981; Guri et al., 1988). Shigyo et al., (1996) reported *Pgi* isozyme banding patterns using various *Allium* species and hybrid plants; the banding pattern in this study was very similar to their hybrid banding pattern. Therefore, in this paper, we tentatively regarded as case (1) the *Pgi* bands shown in *Pgi-1* genotype 'bb' or 'ab.' The triple-band pattern indicates two homodimer bands and one heterodimer, as PGI is a dimeric enzyme. The homodimer 'aa' in *Pgi-1* was not observed in any accession. In addition, accessions with heterodimers were observed only in Southeast and East Asia accessions. These three isozyme loci provided variable band patterns, namely, three *Lap-1* patterns, six *Lap-2* patterns, and two *Pgi-1* patterns. From these combinations, there are, theoretically, 36 isozyme genotypes possible;

however, only 15 genotypes were observed in this study. The numbers of genotypes found in each regional group are shown in Table 9. Accessions possessing an ‘aa’ band in *Lap-1* were seen in the Central Asia and Northern Mediterranean groups, and they did not have an ‘ab’ band in *Pgi-1*.

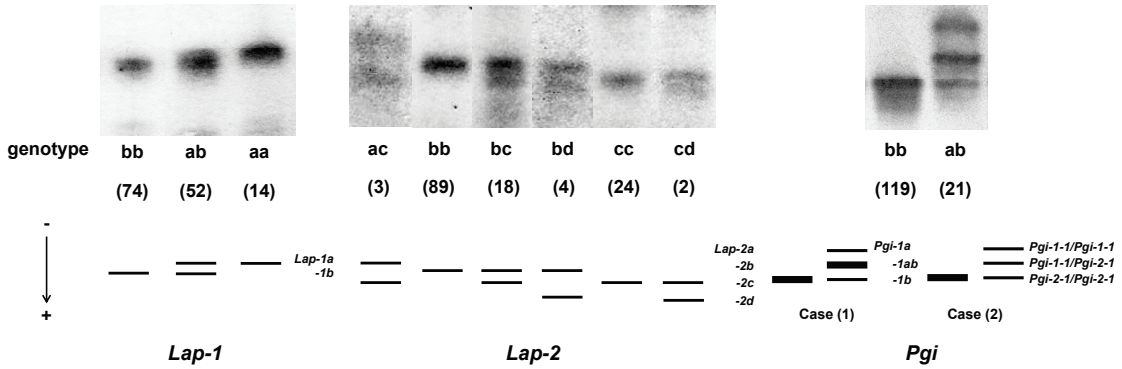

Fig. 10 Isozyme zymograms and genotypes of the *Lap-1*, *Lap-2* and *Pgi-1* enzyme systems. Numbers in parentheses represent the number of accessions. In *Pgi*, two cases were presumed, case (1); there is single gene in garlic, and case (2); there are two monomorphic genes and they form heterodimers.

Table 9. Determined 15 groups based on the observation of isozyme genotype and number of groups in each origin of accessions

Isozyme	Genotype			Central Asia	Northen Mediterranean	Southeastern Mediterranean	China	Japan		Southeast Asia	The New World	Unknown
genotype	*Lap-1*	*Lap-2*	*Pgi-1*	(n = 29)	(n = 13)	(n = 14)	(n = 10)	Group A (n = 31)	Group B (n = 18)	(n = 16)	(n = 6)	(n = 3)
1	aa	ac	bb	1	2	0	0	0	0	0	0	0
2	aa	bb	bb	2	1	0	0	0	0	0	0	0
3	aa	bc	bb	5	1	0	0	0	0	0	0	0
4	aa	cc	bb	2	0	0	0	0	0	0	0	0
5	ab	bb	ab	0	0	0	0	2	0	0	0	0
6	ab	bb	bb	1	3	2	2	4	2	2	2	2
7	ab	bc	bb	6	4	0	0	0	0	0	0	0
8	ab	bd	bb	1	1	0	2	0	0	0	0	0
9	ab	cc	bb	4	1	9	0	0	0	0	0	1
10	ab	cd	bb	1	0	0	0	0	0	0	0	0
11	bb	cd	bb	1	0	0	0	0	0	0	0	0
12	bb	bb	ab	0	0	0	1	3	5	10	0	0
13	bb	bb	bb	3	0	1	5	22	9	2	4	0
14	bb	bc	bb	1	0	0	0	0	0	0	0	0
15	bb	cc	bb	1	0	2	0	0	2	2	0	0

Group A; collected from Honshu, Group B; collected from islands in Western Japan

Allelic frequencies and heterozygosity

Allelic frequencies showed different tendencies among groups (Fig. 11). *Lap-1a* appeared mainly in the Central Asia (58.6%) and Northern (65.4%) groups, while in the China, Japan, and Southeast Asia groups, its frequency was low. Japan Group A showed an especially low frequency (9.7%), although it is located at the same latitude as the Central Asia group (approximately 40 °N). Thus, high *Lap-1a* frequency was specific to the Central Asia and Northern Mediterranean groups. In other groups, *Lap-1b* frequency was high (more than 40%). Regarding *Lap-2*, four alleles (*-2a*, *-2b*, *-2c*, and *-2d*) were observed in the Central Asia and Northern Mediterranean groups, while one or two alleles (*-2b* and *-2c*) were observed in other groups. Groups from Japan, China, Southeast Asia (the eastern side of Central Asia), and the New World showed mainly *-2b*. Moreover, the frequency of *Lap-2c* in the Central Asia and Northern and Southeastern Mediterranean groups was high, and its frequency tended to decrease toward the east. Allele *-2a* was seen in the Northern Mediterranean and Central Asia groups, and allele *-2d* was shared across groups from the Northern Mediterranean to China. In *Pgi-1*, almost all groups showed *-1b*. Allele *-1a* was only seen in the China, Japan, and Southeast Asia (especially in Southeast Asia) groups. In each group, the allelic frequencies of isozyme loci were compared to the average of the whole by chi-square testing to detect their deviation from the average of the whole. There were no significant deviations in the frequencies of *Lap-1* and *-2* from the average as a whole. However, in *Pgi-1*, there were significant deviations in the Central Asia, new World, and Northern and Southeast Mediterranean groups (Table 10).

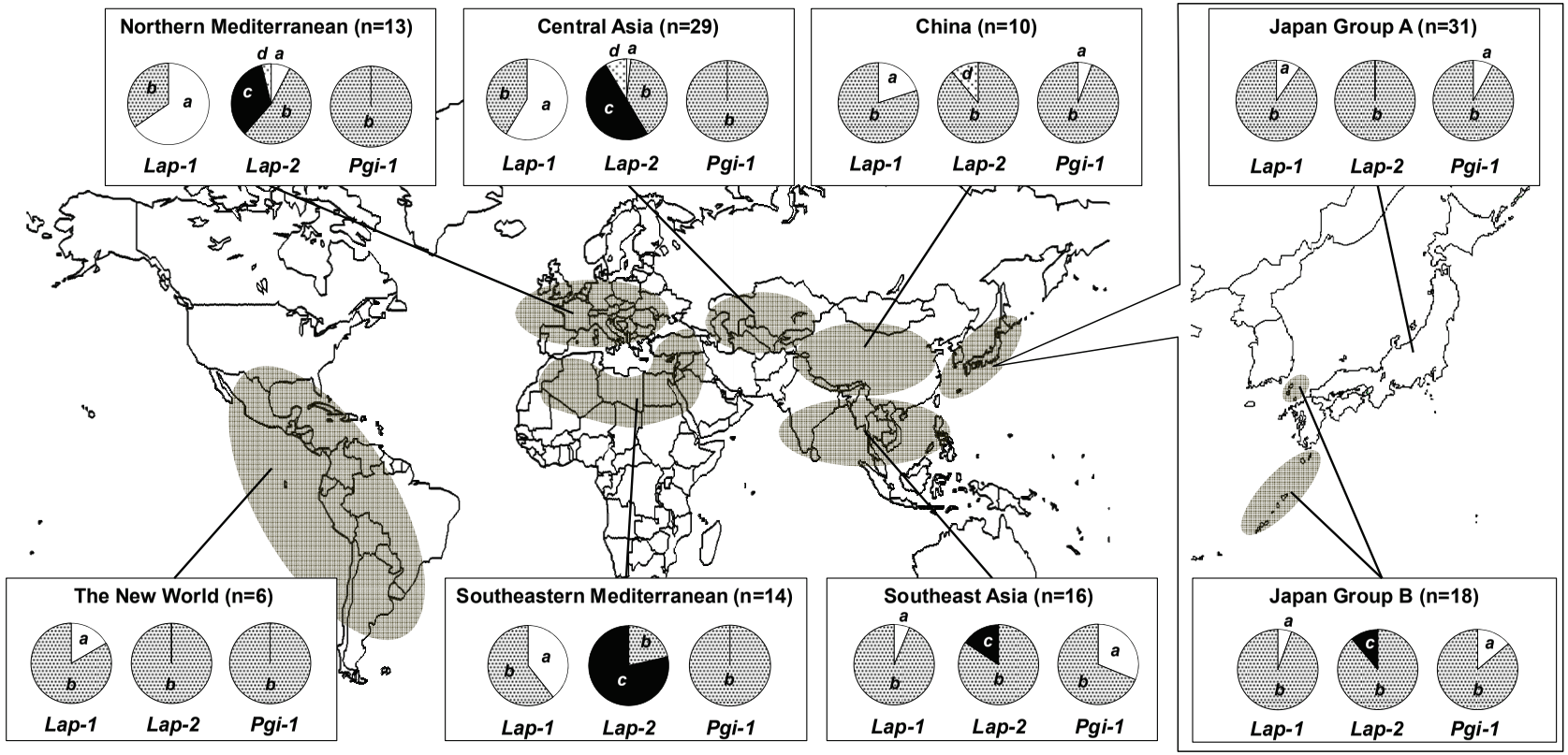

Fig. 11 The allelic frequencies in *Lap-1*, *Lap-2* and *Pgi-1* isozyme loci at various regions. Numbers in parentheses represent the number of accessions.

Table 10. Allelic frequencies in various origins of garlic accessions.

Locus	Allele	Whole plant	Central Asia	Northen Mediterranean	Southeast Mediterranean	China	Japan		Southeast Asia	The New World
							Group A[b]	Group B		
	N	140[a]	29	13	14	10	31	18	16	6
Lap-1	*a*	0.286	0.586	0.654	0.393	0.200	0.097	0.056	0.063	0.167
	b	0.714	0.414	0.346	0.607	0.800	0.903	0.944	0.938	0.833
Lap-2	*a*	0.011	0.017	0.077	0.000	0.000	0.000	0.000	0.000	0.000
	b	0.714	0.397	0.538	0.214	0.889	1.000	0.889	0.844	1.000
	c	0.254	0.500	0.346	0.786	0.000	0.000	0.111	0.156	0.000
	d	0.021	0.086	0.038	0.000	0.111	0.000	0.000	0.000	0.000
Pgi-1	*a*	0.075	**0.000**[c]	**0.000**	**0.000**	0.056	0.081	0.139	0.313	**0.000**
	b	0.925	**1.000**	**1.000**	**1.000**	0.944	0.919	0.861	0.688	**1.000**

[a] Whole plant contains unknown accessions.

[b] Goup A and B denote garlic accessions collected from Honshu and islands in Western Japan, respectively.

[c] Bold letter indicates significant deviations in allelic frequencies by Chi-square testing as compared to the average of the whole.

The observed (H_o) and expected (H_e) heterozygosity in each locus ranged from 0.111 to 0.786 and 0.105 to 0.585 in *Lap-1*, from 0.000 to 0.615 and 0.000 to 0.583 in *Lap-2*, and from 0.000 to 0.625 and 0.000 to 0.430 in *Pgi-1*, respectively (Table 11). The means over loci in each group ranged from 0.111 to 0.436, and the average was 0.234. In *Lap-1*, the Central Asia and Northern and Southeastern Mediterranean groups had higher levels of heterozygosity than did other groups. The *Lap-2* tendency was similar to that of *Lap-1*, but the heterozygosity was zero in the Southeastern Mediterranean group. In *Pgi-1*, the Southeast Asia group showed a high level of heterozygosity. This demonstrated that the Central Asia, Southeast Asia, and Northern and Southeastern Mediterranean groups had large amounts of genetic diversity. According to chi-square testing, some loci deviated significantly ($p < 0.05$) from the Hardy–Weinberg equilibrium (*Lap-1* and *Lap-2* loci in the Southeastern Mediterranean group and a *Lap-1* locus in the Southeast Asia group and Japan Group B). The G_{st} values for *Lap-1*, *Lap-2*, and *Pgi-1* were 0.222, 0.336, and 0.133, respectively. The overall G_{st} value was 0.259. This score indicates that about 26% of the total genetic variation was derived from genetic differentiation.

Table 11. Genetic variability at three isozyme locus in each origin of accessions.

Isozyme locus	Groups	Whole	Central Asia (n=29)	Northen Mediterranean (n=13)	Southeastern Mediterranean (n=14)	China (n=10)	Japan Group A (n=31)	Japan Group B (n=18)	Southeast Asia (n=16)	The New World (n=6)
Lap-1	N_a	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
	H_o	0.386	0.448	0.692	0.786	0.400	0.194	0.111	0.125	0.333
	H_e	0.310	0.552	0.453	0.477	0.320	0.175	0.105	0.117	0.278
	dHWE		ns	ns	*	ns	ns	ns	ns	ns
Lap-2	N_a	2.3	4.0	4.0	2.0	2.0	1.0	2.0	2.0	1.0
	H_o	0.171	0.552	0.615	0.000	0.200	0.000	0.000	0.000	0.000
	H_e	0.260	0.561	0.583	0.337	0.180	0.000	0.198	0.219	0.000
	dHWE		ns	ns	***	ns	Monomorphic	***	***	Monomorphic
Pgi-1	N_a	1.5	1.0	1.0	1.0	2.0	2.0	2.0	2.0	1.0
	H_o	0.146	0.000	0.000	0.000	0.100	0.161	0.278	0.625	0.000
	H_e	0.115	0.000	0.000	0.000	0.105	0.148	0.239	0.430	0.000
	dHWE		Monomorphic	Monomorphic	Monomorphic	ns	ns	ns	ns	Monomorphic
Mean H_o over loci		0.234	0.333	0.436	0.262	0.233	0.118	0.130	0.250	0.111

N_a; number of alleles, H_o; observed heterozygocity, H_e; expected heterozygocity,

dHWE; deviations from Hardy-Weinberg equilibrium (ns = not significant, * = $p < 0.05$, *** = $p < 0.001$)

The obtained data of morphological and physiological traits of garlic were subjected to principal component analysis (PCA) to examine their relationship to their geographical origin. Three PCs were obtained, accounting for 84.2% of the total variance. PC1 represented 43.1% and was strongly related to bulb weight, bulb diameter, number of cloves, and clove weight; PC2 represented 27.5% and was positively related to scape length, number of bulbils, and bolting period; PC3 represented 13.6% and was strongly related to the number of cloves (Table 12). Moreover, scatter plots were made from the obtained scores, and all plots were given isozyme groups as shown in Table 9 in order to evaluate their association with several garlic traits. A 2D scatter plot of the PCA scores for PC1 and PC2 principal components is shown in Fig. 12. Of the 107 accessions, only 66 accessions are plotted in Fig. 12 because no data were available for some accessions (especially accessions from the Southeastern Mediterranean and Southeast Asia groups, which possess incomplete bolting or non-bolting types). However, PCA could divide garlic accessions into several groups. Central Asia and Northern Mediterranean accessions were separated from East and Southeast Asia accessions. Accessions from Central Asia and the Northern Mediterranean located in high-latitude regions (40 °N–45 °N) had elongated scapes, produced many bulbils, and matured late. However, accessions from middle- to low-latitude regions (15 °N–30 °N) developed short scapes, produced small bulbils and heavy bulbs, and matured early. Meanwhile, isozyme genotypes 3, 4, 7, and 8, which possessed homozygous ‘aa’ in *Lap-1* or were heterozygous in *Lap*-2, were found in the Central Asia and Northern Mediterranean groups, while genotypes 6, 9, 12, 13, and 15, which possessed homozygous ‘bb’ in *Lap-1* and ‘bb’ or ‘cc’ in *Lap-2*, were found in other groups (Table

9). Moreover, based on MANOVA tests, there were several significant differences in garlic traits between isozyme genotypes and the groups of origin (Table 13).

Table 12. Pearson's correlation coefficients between PCs and garlic traits.

Traits	PC 1	PC 2	PC 3
Scape length (cm)	0.200	0.868	-0.171
Number of bulbils	0.092	0.895	-0.127
Bulb weight (g)	0.960	0.090	0.069
Bulb diameter (cm)	0.981	0.010	0.057
Number of cloves	0.371	0.155	0.908
Clove weight (g)	0.826	-0.143	-0.445
Bolting period (day)	-0.517	0.566	0.090

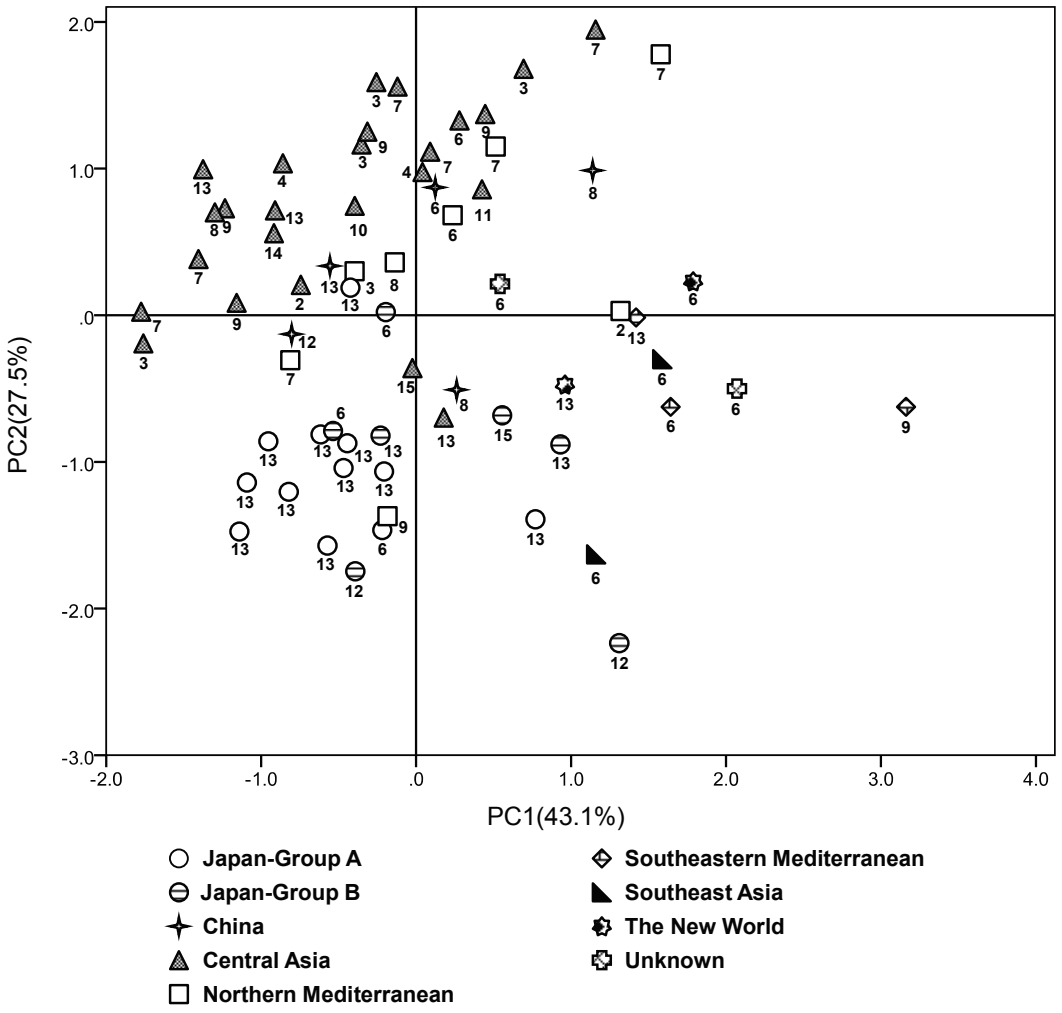

Fig. 12 Plot of the first and second principal components obtained from garlic morphological and physiological data. PC1 and PC2 accounted for 43.1 and 27.5% of the total variation, respectively. The numbers under the plots denote isozyme groups determined in Table 9.

Table 13. Multivariate analysis of variance (MANOVA) test for the several garlic traits in isozyme genotypes and origin of accessions.*indicates significantly different at $p < 0.05$.

Traits	Factor	Sum of square	degree of freedom	Mean square	F value	Significant level	
bulbils	*lap-1*	985.977	2	492.989	0.708	0.496	
	lap-2	8194.552	5	1638.910	2.355	0.050	
	pgi-1	411.752	1	411.752	0.592	0.445	
	region	12182.925	8	1522.866	2.188	0.039	*
	lap-1 × *lap-2*	5011.772	6	835.295	1.200	0.317	
	lap-1 × *pgi-1*	0.523	1	0.523	0.001	0.978	
	lap-1 × region	13132.361	6	2188.727	3.145	0.009	*
	lap-2 × region	11853.477	7	1693.354	2.433	0.028	*
	pgi-1 × region	159.046	2	79.523	0.114	0.892	
	Error	51306.839	75	684.091			
Scape length	*lap-1*	553.457	2	276.728	0.412	0.664	
	lap-2	12150.636	5	2430.127	3.621	0.006	*
	pgi-1	937.987	1	937.987	1.397	0.241	
	region	12460.413	8	1557.552	2.321	0.029	*
	lap-1 × *lap-2*	3126.479	6	521.080	0.776	0.591	
	lap-1 × *pgi-1*	80.778	1	80.778	0.120	0.730	
	lap-1 × region	11987.552	7	1712.507	2.551	0.021	*
	lap-2 × region	7136.123	9	792.903	1.181	0.321	
	pgi-1 × region	2467.793	3	822.598	1.226	0.307	
	Error	46983.411	70	671.192			
bolting period	*lap-1*	2737.058	2	1368.529	0.336	0.716	
	lap-2	5049.317	4	1262.329	0.310	0.870	
	pgi-1	178.012	1	178.012	0.044	0.835	
	region	113860.636	8	14232.579	3.497	0.003	*
	lap-1 × *lap-2*	2928.436	6	488.073	0.120	0.994	
	lap-1 × *pgi-1*	29113.899	1	29113.899	7.154	0.010	*
	lap-1 × region	57209.474	6	9534.912	2.343	0.043	*
	lap-2 × region	33875.032	7	4839.290	1.189	0.325	*
	pgi-1 × region	20114.040	2	10057.020	2.471	0.094	
	Error	219759.143	54	4069.614			
Clove weight	*lap-1*	1.755	2	0.878	1.078	0.347	
	lap-2	7.211	5	1.442	1.771	0.134	
	pgi-1	3.875	1	3.875	4.758	0.033	*
	region	3.625	8	0.453	0.556	0.809	
	lap-1 × *lap-2*	3.180	6	0.530	0.651	0.689	
	lap-1 × *pgi-1*	0.317	1	0.317	0.389	0.535	
	lap-1 × region	5.773	6	0.962	1.182	0.329	
	lap-2 × region	16.257	7	2.322	2.852	0.013	*
	pgi-1 × region	0.692	2	0.346	0.425	0.656	
	Error	45.608	56	0.814			

Discussion

Maass and Klaas (1995) classified 300 garlic accessions from the Old World into four subgroups using isozymes and RAPD, but they did not fully evaluate the morphological characteristics due to growing conditions. In this study, we could evaluate the morphological and physiological traits in Japanese climate conditions as well as the isozyme variations of garlic accessions collected worldwide. In this study, groups of garlic accessions showed various morphological and physiological variations based on their origins. There are two possible reasons for these differences: (1) mutations have accumulated through the domestication process in each region; or (2) mutations had already occurred in the ancestral population. There have been some reports of intraspecific variation among cultivated garlic landraces in Iran (Shaaf et al. 2014), Tunisia (Jabbes et al., 2012), Brazil (Buso et al., 2008), and China (Chen et al., 2013). The variations found in this study probably would be derived from standing variations or mutations caused by adaptation, hitchhiking, or genetic drift in the process of garlic's domestication before its cultivation. Etoh (1985) supported a hypothesis that garlic has evolved from fertility to sterility and from a complete bolting type to a non-bolting type through an incomplete bolting type. Developing bulb-related traits in the Southeastern Mediterranean group were superior to those in the other groups, although the bolting period of that group was not significantly different from that of other groups. However, many Southeastern Mediterranean accessions bolted incompletely or did not bolt. Garlic is frequently called a medium-temperature plant because it grows well in medium temperatures (Etoh 1985). Etoh (1985) also reported

that the Mediterranean climate (cold in the winter, hot and dry in the summer) is suitable for growing garlic. In this plant, bulbil formation at the top of the scape causes a decrease in bulb yield due to competition with the bulbs for nutrients (Etoh 1985, Hong and Etoh 1996). Additionally, geographical conditions accelerated garlic selection. Etoh (1985) suggested that garlic collected from areas with harsh, cold winters with heavy snow, such as Northern Europe, Northern America, and Northern Japan (high latitude areas), have evolved to be non-bolting because of the severe agroclimatic conditions. Both non- and incomplete-bolting traits are presumably the products of adaptation to unfavorable climatic conditions (Etoh 1985). Specifically, garlic clones might have ceased bolting due to farmers' efforts to avoid decreased bulb yields. In a tropical area, however, other traits were required. Etoh and Simon (2002) reported that many tropical garlic cultivars develop only light bulbs because the differentiation of axillary buds and their development into cloves require low temperatures. In South Asia, garlic leaves are consumed as a green vegetable, and special clones have been selected for leaf production. Etoh and Simon (2002) stated that selection for leaf-producing rather than bulb-producing plants may have occurred in warm or hot regions.

In order to discuss the sources of the present variations of garlic, we assume the followings two hypotheses: (1) domestication with some artificial selection occurred in Central Asia (these populations may have standing variations) and widely spread to other regions; or (2) domesticated garlic expanded to other regions of the world with accumulating mutations. In these cases, there are two possibilities: (1) the sources of local adaptation and artificial selection are derived from standing variations; or (2) the sources of local adaptation and artificial selection are derived from mutations accumulated during the expansion. Therefore, it has been assumed that ancestral

domesticated garlic populations have adapted in various regions using standing variations or mutations accumulated during the expansion, evolving with human-preferred traits over a long history of cultivation.

Isozyme loci showed that polymorphisms and allelic frequencies were different among the regional groups of accessions. In this study, only 15 isozyme genotypes were observed (Table 9). Central Asia has all genotypes except genotypes 5 and 12 (containing 'ab' banding pattern in *Pgi-1*), while other groups have only a few specific genotypes. This is probably due to linkage disequilibrium caused by regional differentiation or other factors. Central Asia and Northern and Southeastern Mediterranean accessions showed high heterozygosity. On the other hand, accessions from Japan, China, Southeast Asia, and the New World showed low levels of heterozygosity. Especially, the lowest H_o among groups was seen in the New World group. This is probably due to the small population size or other factors, such as selective sweeps at those loci and the founder effect. The Northern and Southeastern Mediterranean groups showed tendencies different from those of the Asian groups. This result is in agreement with previous reports (Etoh 1985; Pooler and Simon 1993; Maass and Klaas 1995). Chi-square testing showed no significant differences in allelic frequencies as compared to the average of the whole, except for *Pgi-1*. However, from the G_{st} scores, genetic differentiation between regions was expected to be high. Additionally, some loci deviated significantly ($p < 0.05$) from the Hardy–Weinberg equilibrium (*Lap-1* and *Lap-2* loci in the Southeastern Mediterranean and the *Lap-1* locus from Japan Group B and Southeast Asia) (Table 11). Some factors are considered to disturb the HWE (e.g., genetic drift, migration, natural or artificial selection, and non-random mating). In these groups, there is a possibility that these factors affect the

allelic frequencies. Kazakova (1971) reported that the Mediterranean region (from the west side of the Tien Shan Mountains to the Caucasus) contains a mix of fertile and sterile garlic. This region is regarded as garlic's secondary center of origin. In other words, ancestral garlic was widespread in this area, and domestication was begun. Then, it is believed that a random mating population stopped random mating and started clonal reproduction. Thus, there is a possibility that garlic from the Southeastern Mediterranean has been affected by selective pressure for human needs (such as superior bulb formation) during a long cultivation history. Japan Group B (collected from islands in Western Japan) also has a history of varietal establishment. For example, accession 65 "Iki-shu" was originally introduced from Jejudo Island in Korea to Iki Island in Japan. Thus, it probably originated from a local clone in Jejudo Island (Etoh 1985). Information about the origins of accessions in this region is limited and complicated. It is likely that many kinds of garlic clones from surrounding countries were introduced to this region. Alternatively, accessions in this region might have been strongly affected by selection for human-preferred traits (such as high adaptability to agroclimatic conditions). In Southeast Asia, it is estimated that garlic was introduced from the Mediterranean to India more than 5,000 years ago and then spread to this region (Etoh and Simon 2002). Maass and Klaas (1995) inferred that garlic in this area might have originated from *A. longicuspis* a long time ago through India, after acquiring special adaptations for various climatic stressors (such as heat, desert, strong sunshine, and disease) necessary for its spread through the tropics. It is also possible that these stressors might have resulted in natural selection. Meanwhile, the *Pgi-1* genotype 'aa' was not observed in any accessions, not even in the Central Asia group. On the other hand, *Pgi-1* 'ab' was observed mainly in the Southeast Asia group. It is possible that

garlic possessing an ‘aa’ genotype in *Pgi-1* might exist in the region from India to Southeast Asia. Alternatively, some garlic from the ancestral population in this area formed intergenic heterodimers and expanded to South Asia. Further investigation is needed to confirm genetic variations in India and the surrounding areas.

PCA analysis showed relationships between morpho-physiological traits and isozyme genotypes. According to MANOVA tests, there were significant differences in some traits among isozyme genotypes; however, it suggested that geographical factors also have significance (Table 13). Thus, it was expected that the significant associations of alleles with traits were caused by regional differences. To reveal the relationships between genetic structures and some traits, further analyses with many loci, such as those using microsatellites or others, are necessary.

Genetic changes or different combinations of genes after garlic’s exposure to different agroclimatic conditions would result in different phenotypes. Geographical (latitude) information could explain the selection of garlic based on bolting traits. Therefore, adaptation and selection in garlic seem to depend on various environmental conditions and human preferences. In this study, garlic accessions showed great diversity of morpho-physiological traits and isozymes. Other diversity studies have been carried out regarding the variability of chemical production in a set of garlic collections such as organosulfur compounds (Kamenetsky et al. 2005; Hornickova et al. 2009; Ovesna et al. 2011; Jabbes et al. 2012) or phenolic compounds (Lu et al. 2011), which have benefits for human health. In previous chapters, we demonstrated the association between bio-morphological traits (bolting types and chemical production levels mentioned above) and geographical distribution. It is possible that our materials are diverse not only in their visible traits but also in their DNA or other chemical production

levels. Moreover, Kamenetsky et al. (2005) stated that garlic from the place of origin possesses superior traits, such as tolerance to disease and pests and better adaptation to biotic or abiotic stress, than are seen in current cultivars. Further research on the genetic structure of garlic populations is necessary to utilize new breeding materials for future marker-assisted garlic breeding programs.

Chapter 5: EVALUATION OF GROWTH CHARACTERISTICS OF GARLIC IN SAND-ARID FIELDS

Introduction

Garlic has a long history of vegetative propagation. The long selection process of garlic has resulted in its high adaptation in various regions. However, this could be caused by the loss of several characteristics. Kamenetsky et al. (2005) made the point that garlic from the place of origin possesses superior traits, such as tolerance to disease and pests and better adaptation to biotic or abiotic stress, than are seen in current cultivars. There is a high possibility of finding garlic that has superior traits against abiotic stress.

In this chapter, we investigate the growth characteristics of garlic accessions in arid climate conditions.

Materials and Methods

Plant materials

Bulbs of 105 garlic accessions collected from around the world since the 1970s have been managed at Yamaguchi University, Japan (34.14ºN, 131.47ºE). In addition, bulbs of 33 garlic accessions were managed at Saga University, Japan (33.24ºN, 130.29ºE) until 2013, when management of these collections was taken over by Yamaguchi University. The following 8 groups were categorized based on their origins: 23 accessions from Honshu, Japan (Group A); 16 accessions from islands in Western

Japan (Group B); 7 accessions from China; 11 accessions from Southeast Asia; 25 accessions from Central Asia; 8 accessions from the Northern Mediterranean; 9 accessions from the Southeastern Mediterranean; and 4 accessions from the New World. Thus, a total of 104 accessions were used in this chapter (Table 14). These bulbs were obtained from local markets or national institutions in each country. Detailed information regarding some accessions was reported by Etoh (1985, 1986), Hong et al. (2000), and Etoh et al. (2001). These bulbs were stored at 4°C in dark conditions in the summer.

The garlic accessions were planted in an experimental arid-land research field at Tottori University (35.53ºN, 134.21ºE) at the end of October 2013 (Fig. 13). A compound fertilizer was applied before planting. During the growing season, 4 cloves of a uniform size per accession were randomly selected from the bulbs and were grown in rows 20 cm apart. Liquid fertilizer was supplied every week. All garlic accessions were harvested at the end of June and cured (completely dried of leaves and outer skins in a vented greenhouse) in Yamaguchi. We attempted to determine which of these accessions were highly adaptable. The same trials were carried out in 2014–2015 using 80 garlic accessions, including highly adaptable accessions, with a plastic greenhouse experimental plot. In adaptable accessions, some chemical production levels in the bulbs were analyzed.

Table 14. Garlic accessions used in this chapter.

No.	Collected country or site	Managing number or name	Collected country or site	Accession information	Remarks column
1	Japan - Group A	6	Japan	Etoh 1985	''Niigata-Sado''
2		8	Japan	Etoh 1985	''Ibaraki''
3		15	Japan	Etoh 1985	''Hamamatsu''
4		37	Japan	Etoh 1985	''Okute-B''
5		40	Japan	Etoh 1985	''Kokotsu''
6		56	Japan	Etoh 1985	''California Early''
7		75	Japan	Etoh 1985	''Kushikino-wase''
8		360	Japan	-	''Hiru''
9		Hirado	Japan	-	''Hirado''
10		Chugokukei ninniku	Japan	-	-
11		Chiba-shoukyu	Japan	Saga university, Japan	-
12		Enhei	Japan	Saga university, Japan	-
13		Enshuu-gokuwase	Japan	Saga university, Japan	-
14		Kagoshima	Japan	Saga university, Japan	-
15		Kashu-wase	Japan	Saga university, Japan	-
16		Kashu-okute	Japan	Saga university, Japan	-
17	Japan - Group B	Katish(touhiru)	Japan	Saga university, Japan	-
18		Kouchi-shoukyuu	Japan	Saga university, Japan	-
19		Nagano	Japan	Saga university, Japan	-
20		Saga-ooninnniku	Japan	Saga university, Japan	-
21		S61-tashirouetuke	Japan	Saga university, Japan	-
22		Setouchi	Japan	Saga university, Japan	-
23		Wase-ninniku	Japan	Saga university, Japan	-
24		65	Japan	Etoh 1985	''Iki-shu''
25		67	Japan	Etoh 1985	''Amami-A''
26		68	Japan	Etoh 1985	''Amami-B''
27		129	Japan	Etoh 1985	''Iriomote''
28		501	Japan	-	''Tarama''
29		540	Japan	-	-
30		Okinawa(naha)	Japan	-	''Naha''
31		Okinawa(tamagusuku)	Japan	-	''Tamagusuku''
32		Okinoerabu	Japan	Saga university, Japan	-
33		Kikai(oodama)	Japan	Saga university, Japan	-
34		Kikai(ikumi)	Japan	Saga university, Japan	-
35		Kikai(onozu)	Japan	Saga university, Japan	-
36		Iki-ooninnniku	Japan	Saga university, Japan	-
37		Iki-wase	Japan	Saga university, Japan	-
38		Okinawa-nanbu	Japan	Saga university, Japan	-
39		Taishu-san	Japan	Saga university, Japan	-
40	China (30°N-40°N)	54	China	Etoh 1985	''Fukushu (Foochow,China)''
41		362	China	Hong and Etoh 1996	''Urumchi''
42		397	China	Hong and Etoh 1996	''Kashgar''
43		524	China	-	''Guizhou-D''
44		Kankousan	China	Saga university, Japan	-
45		Hongkong-wase	China	Saga university, Japan	-
46		Shang-hai	China	Saga university, Japan	-
47	Southeast Asia (15°N-20°N)	39	Taiwan	-	''Seira''
48		45	Taiwan	Etoh 1985	''Taiwan-shokyu-pinku''
49		180	Taiwan	-	''Taipei''
50		Mai Dinh	Vietnam	-	''Mai Dinh''
51		IIT	India	-	-
52		Chang Mai small	Thailand	-	-
53		Chang Mai large	Thailand	-	-
54		67-4	Thailand	Saga university, Japan	-
55		151-1	Thailand	Saga university, Japan	-
56		16-5	Thailand	Saga university, Japan	-
57		210-3	Thailand	Saga university, Japan	-

Table 14. Continued.

No.	Collected country or site	Managing number or name	Collected country or site	Accession information	Remarks column
58	Central Asia	199	Frunze	Etoh 1986	‘‘Frunze-2’’
59		F17	Cenrtal Asia	-	-
60		F30	Cenrtal Asia	-	-
61		F31	Cenrtal Asia	-	-
62		F112	Cenrtal Asia	Hong et al. 2000	-
63		F115	Cenrtal Asia	Hong et al. 2000	-
64		F117	Cenrtal Asia	Hong et al. 2000	-
65		F138	Cenrtal Asia	Hong et al. 2000	-
66		F146	Cenrtal Asia	Hong et al. 2000	-
67		F147	Cenrtal Asia	Hong et al. 2000	-
68		F189	Cenrtal Asia	Hong et al. 2000	-
69		F215	Cenrtal Asia	Hong et al. 2000	-
70		F227	Cenrtal Asia	Hong et al. 2000	-
71		F424	Cenrtal Asia	Hong et al. 2000	-
72		F436	Cenrtal Asia	Hong et al. 2000	-
73		F1-200-23	Cenrtal Asia	Hong et al. 2000	-
74		F1-200-34	Cenrtal Asia	Hong et al. 2000	-
75		F1-200-92	Cenrtal Asia	Hong et al. 2000	-
76		Fs405	Cenrtal Asia	Hong et al. 2000	-
77		Fs407	Cenrtal Asia	Hong et al. 2000	-
78		Fs410	Cenrtal Asia	Hong et al. 2000	-
79		Fs414	Cenrtal Asia	Hong et al. 2000	-
80		Fs422	Cenrtal Asia	Hong et al. 2000	-
81		Fs424	Cenrtal Asia	Hong et al. 2000	-
82		Kazakhstan	Cenrtal Asia	-	‘‘Chimkent’’
83	Northern Mediterranean	225	Spain	-	‘‘Spain-1’’
84		307	Greek	-	‘‘Thessaloniki market-1’’
85		434	Spain	Etoh et al. 2001	‘‘Spanish Gene Bank’’
86		445	Spain	Etoh et al. 2001	‘‘Spanish Gene Bank’’
87		462	Portugal	Etoh et al. 2001	‘‘Portuguese Gene Bank’’
88		469	Portugal	-	‘‘Braga Gene Bank’’
89		552	Germany	Germany IPK collection All 130	-
90		556	Germany	Germany IPK collection All 1035	-
91	Southeastern Mediterranean	55	Egypt	Etoh 1985	‘‘Egypt’’
92		489	Egypt	-	‘‘Egypt-2’’
93		490	Egypt	-	‘‘Egypt-3’’
94		493	Syria	-	‘‘Syria-1’’
95		542	Turkey	-	-
96		Egypt	Egypt	-	‘‘Aswan’’
97		Syria-3	Syria	-	-
98		Syria-5	Syria	-	-
99		Gatur	Turkey	-	-
100	The New World	60	Chili	Etoh 1985	‘‘Chili’’
101		69	Columbia	-	-
102		137	Peru	Etoh 1985	‘‘Peru’’
103		Chili	chili	Saga university, Japan	-
104		Columbia	columbia	Saga university, Japan	-

Fig. 13 Growing scenes of garlic accessions in Arid Land Research Center, Tottori University, Japan (35.53ºN, 134.21ºE).

Growing characteristics of garlic in arid conditions

In 2012–2013, about a month after harvest, developed bulbs and roots of the accessions were examined for 4 plants of each accession to determine the highly adaptive garlic accessions. In 2014–2015, bulb weights and roots in fields and in a plastic greenhouse experimental plot were examined to compare their growth characteristics.

Chemical analyses

In 2014–2015, biochemical analyses were conducted on the accessions determined to be highly adaptable. Water and 70% EtOH extraction from bulbs was carried out using 3 bulbs for each.

AlCSO (S-allyl-L-cysteine sulfoxide) and phenolic contents were determined according to the same methods outlined in chapter 2. Fructan content was also determined by the thiobarbituric acid method (Percheron 1962) with minor modifications. To determine fructan alone, sucrose was first removed by digestion with invertase. In addition, free fructose was removed from the extracts by heating an aliquot in 1N NaOH at 100℃ for 10 min. A 20-μL aliquot of extract was incubated with 10 μL of 2 mg/mL invertase (baker's yeast, Sigma-Aldrich, USA) and 10 μL of a 25 mM ammonium acetate buffer (pH 5.5) for 5 min. The fructan contents of garlic were expressed as the inulin equivalent per gram fresh weight (mg/gFW).

Data analysis

All obtained data were used for a one-way analysis of variance (ANOVA), a Tukey's test using SPSS 22.0 software (SPSS Japan, Inc., Tokyo, Japan).

Results and Discussion

Determination of highly adaptable garlic accessions in 2012–2013

Garlic accessions showed various bulb formation levels. All garlic accessions developed bulbs that varied among the accessions from 0.24 to 14.88 gFW, and the average was 4.12 g (n=91). Northern Mediterranean accessions produced significantly heavier bulbs (8.6±3.7 g) than did those of other groups. There was a high correlation between the formed bulb weight and the dry weight of the developed roots (r=0.861). A histogram analysis based on bulb weight was conducted (Fig. 14). The formed bulbs and roots were inferior to those from a field trial test in Yamaguchi: average 12.73 g. However, some accessions formed bulbs vigorously (Fig. 15). The plant roots of these bulbs were more than 30 cm and were put down not only in an upper, dry sand layer but also in a deeper, wet sand layer in the fields. From these results, it was suggested that these accessions develop roots and take in water and nutrients. As explained in chapter 4, we understand that the accessions from the Northern Mediterranean or Central Asia had clearly different traits from those from Japan or other modern cultivars. Finally, we could select highly adaptive garlic accessions in arid land (Table 15). This area has not only desert but also heavy snow and a strong cold window. It was assumed that these climate conditions were more severe than those in other areas. Therefore, the next trials were conducted in a plastic greenhouse plot.

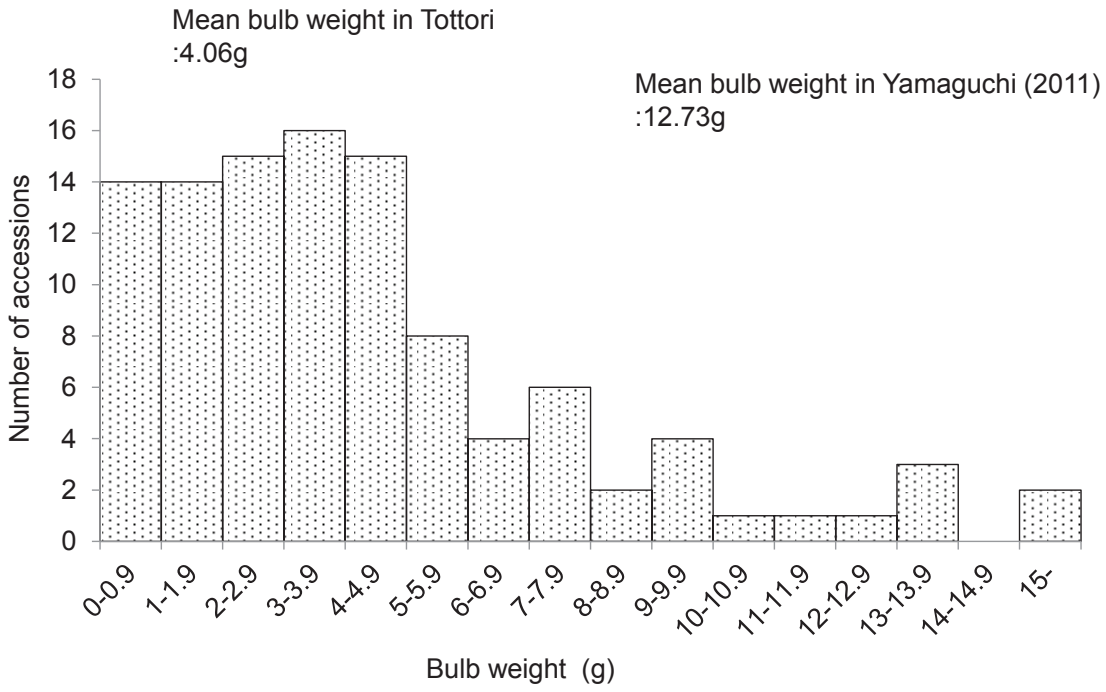

Fig. 14 Histograms of garlic accessions based on bulb weight in 2012-2013.

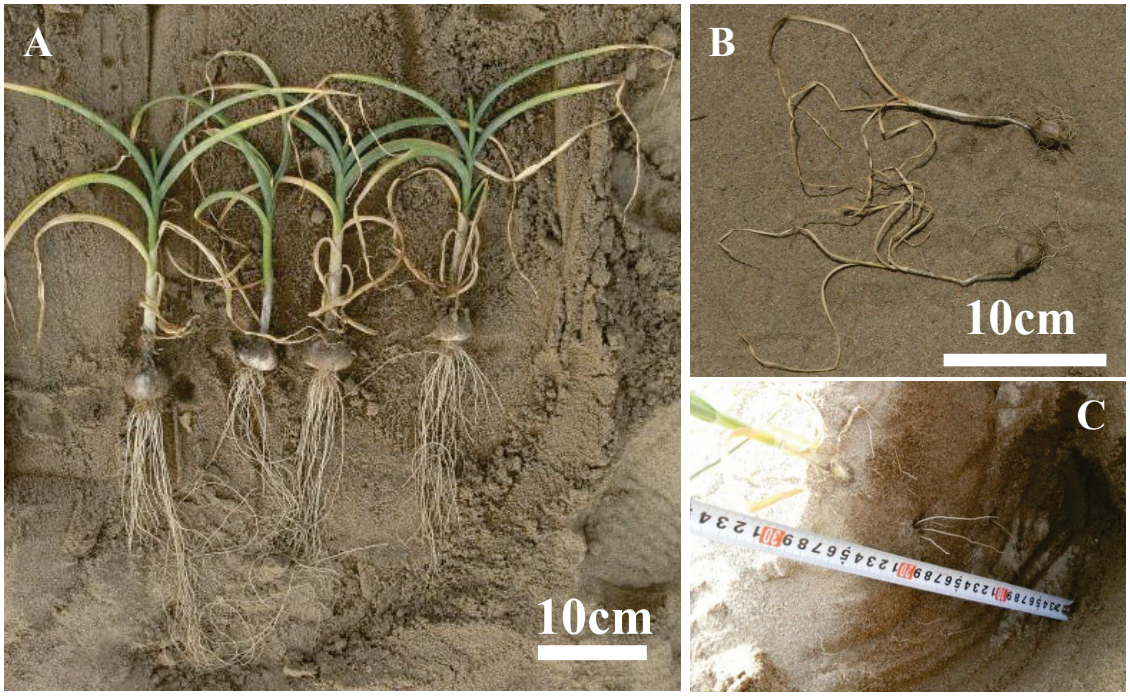

Fig. 15 Harvested garlic accessions. A: accession from Central Asia (Kazakhstan). B: accession from Japan (‘56’) C: vigorous roots development.

Table 15. Determined high-adaptable garlic accessions.

No.	Accession Name	Collected country or site
1	524	Guizhou (China)
2	542	Turkey
3	F436	Central Asia
4	Kikai(ikumi)	Japan
5	F117	Central Asia
6	199	Frunze(Central Asia)
7	F30	Central Asia
8	60	Chili

Growth characteristics in highly adaptable garlic accessions in 2014–2015

In 2014–2015, the same trial was conducted using 80 garlic accessions, including highly adaptable accessions. As shown in Fig 16A in the field plot trial, these garlic growth characteristics were inferior to those in previous years. This is probably because of using a bulb maturing or planting season. In this year, the cloves used were a little smaller than those in previous years. Additionally, the planting season was delayed (October 30). Bulb formation depends on the clove size used and the planting seasons (Etoh 1985). However, in spite of those situations, the plastic greenhouse plot showed vigorous growth traits (Fig. 16B). All garlic accessions of this plot developed bulbs varying from 0.82 to 18.46 gFW. Notably, the highly adaptable accessions found in the previous year varied from 9.28 (accession 199) to 18.46 (accession 524); the average was 12.58 g. Therefore, it was suggested that using a glass greenhouse decreased several growth stresses.

Chemical characteristics in highly adaptable garlic accessions

Meanwhile, the chemical characteristics of highly adaptable garlic accessions were determined (Fig. 17). In AlCSO contents, there were no significant differences between arid conditions (6.03±0.74 mg/gFW in field, 5.43±0.67 mg/gFW in the greenhouse) and normal growth conditions (5.23±0.94 mg/gFW in Yamaguchi). In phenolic contents, both arid land plots showed lower contents than the normal conditions (55.79±3.77 mg/100 gFW in the field, 53.04±3.94 mg/100 gFW in the greenhouse, and 72.64±5.25 mg/100 gFW in Yamaguchi). However, both arid land experimental plots showed significantly higher fructan content than was shown in normal conditions (56.86±4.28 mg/gFW in the field, 56.05±4.49 mg/gFW in the

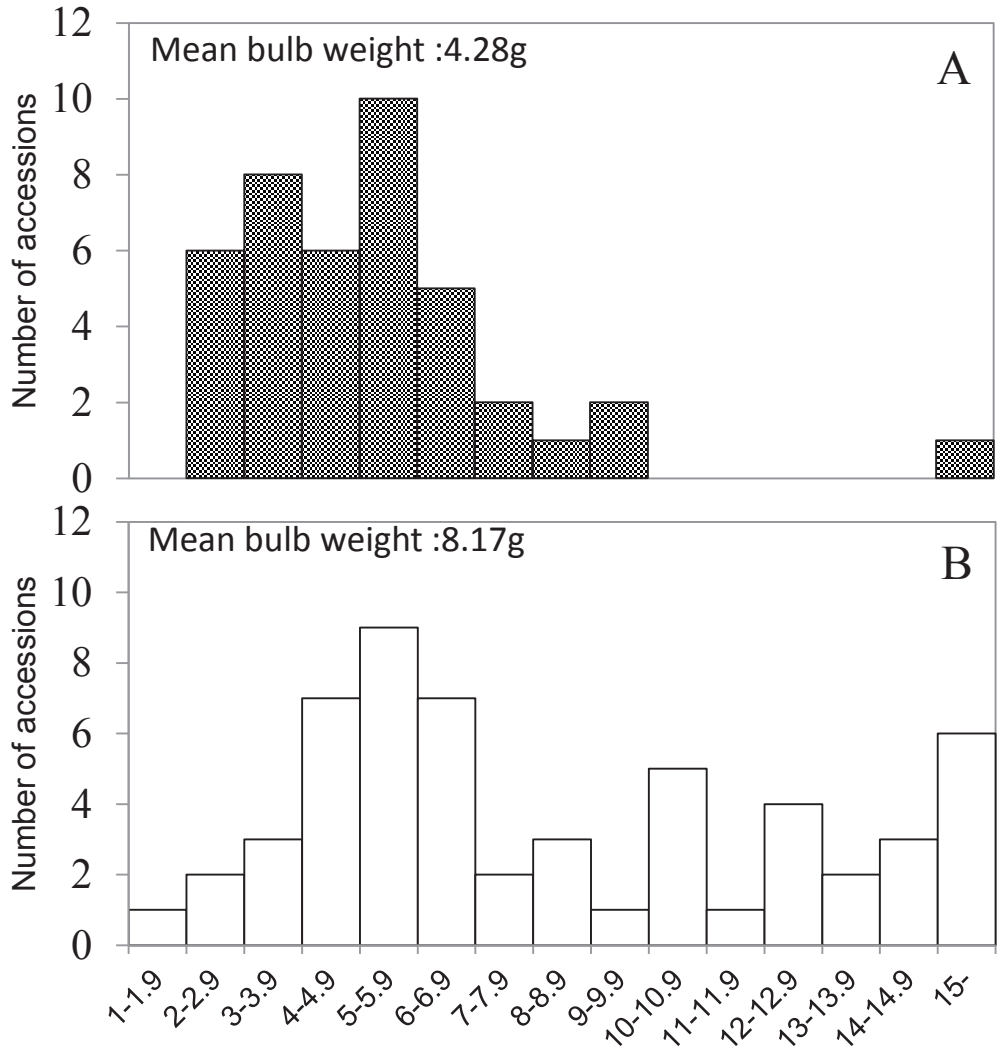

Fig. 16 Histograms of garlic accessions based on bulb weight in 2014-2015.

A: Results in the field plot trial. B: Results in the glasshouse plot trial

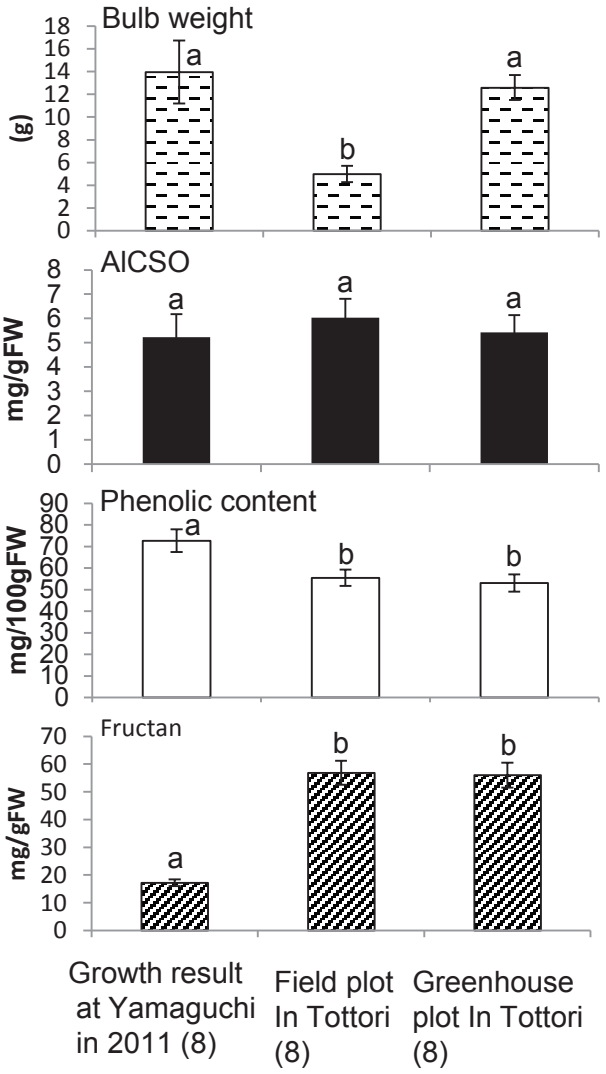

Fig. 17 Comparison of bulb weight and several chemical contents in high-adaptable garlic accessions under different growing conditions. Numbers in parentheses and error bars represent the number of accessions and standard error, respectively. Different letters significant difference by Tukey's test ($p < 0.05$).

greenhouse, and 17.20±1.22 mg/gFW in Yamaguchi). Fructan is a polysaccharide and is suggested to correlate to the drought resistance or cold resistance of a plant (Pilon-Smits 1995). Therefore, it was suggested that these garlic accessions stimulate fructan accumulation systems in order to adapt to arid land climate conditions.

In this research, a large number of accessions died or showed dwarf growing levels. Growing garlic in arid land has probably already been tried. However, *allium* crops that grow well in Japan, such as Rakkyo, bulb onions, or Japanese bunching onions, were selected. Moreover, in bulb onions, more detailed irrigation condition or water stress research was carried out (Kumar et al. 2007). A growth characteristic of garlic in a semi-arid climate in Iran, which has wide arid regions, was surveyed (Abyaneh et al. 2011). However, garlic growth in that arid climate is unknown. The highly adaptable garlic accessions discussed in this chapter have potential for high abiotic stress tolerance against dryness and cold.

In general, abscisic acid (ABA) or salicylic acid (SA) is an important signal molecule modulating plant response to stress. ABA has adjusted stomata and controlled the transpiration of plants to obtain arid tolerance. SA has been shown to play an important role in regulating many physiological processes in plants. Its exogenous application has promoted plant performance under biotic and abiotic stresses (Bideshki et al. 2010; Horvath et al. 2007; Senaratna et al. 2000). However, these chemicals are hormones and are not easily administered. In this research, highly adaptable accessions accumulated fructan content. It has already been reported that environmental factors significantly affect fructan accumulation in various plants (Livingston 2009). The quantification of this chemical is easier than that of both hormones mentioned above. Fructan could become one of the simple chemical markers to confirm abiotic stress

tolerance. Moreover, fructan is also one of the functional components in garlic. Fructan is an important dietary source of fiber that resists digestion (Moshfegh et al. 1999). As a prebiotic, fructan stimulates the growth of beneficial bifidobacteria in the colon (Roberfroid 2005). Therefore, the results of these highly adaptable garlic accessions open the possibility of developing high-value-added garlic production in arid climate conditions.

Chapter 6. GENERAL DISCUSSION

Edible *Allium* species are major crops cultivated worldwide. Garlic (*Allium sativum* L.) is one of the most widely used important cultivated *Allium* species. Garlic is grown in many countries at a wide range of latitudes. For centuries, this plant has been propagated clonally in various regions. As a result, cultivated garlic or clonal lineages exhibit remarkably wide morphological variations. This general discussion refers to the variations observed in this study and offers suggestions regarding the possibility of novel breeding materials.

(1) Association between bio-morphological traits and geographical distribution in garlic collections

Garlic has different morphological traits. One of the most visible traits is bolting. In addition, garlic has a variety of rich chemicals such as organosulfur compounds (Kamenetsky et al., 2005; Hornickova et al., 2009; Ovesna et al., 2011; Jabbes et al., 2012), polysaccharides (Baumgartner et al., 2000), and saponins (Matsuura 2001; Lanzotti 2005; Rivlin et al., 2006; Amagase 2006; Lanzotti et al., 2012).

The present studies have demonstrated the relationship between garlic's bolting type and chemical production levels. Moreover, the bolting types are dependent on garlic's collection site. As mentioned in chapter 2, the non-bolting type of garlic had higher AlCSO and total phenolic contents than did garlic of other bolting types. The AlCSO result was in agreement with those of previous reports (Hornickova et al. 2009).

In addition, in the complete bolting type, the AlCSO content was higher in groups that produced mainly flowers than in groups that produced mainly bulbils. Etoh and Simon (2002) stated that the primitive forms of garlic originally produced umbels with mixed populations of flowers and bulbils. Etoh (1985) supported a hypothesis that garlic evolved from fertility to sterility and from a complete bolting type to a non-bolting type through an incomplete bolting type. Therefore, It is highly probable that garlic was selected naturally or artificially to adapt to environmental conditions in various regions. A long history of cultivation and selection may have resulted in the evolution of garlic from sexual to asexual propagation. Moreover, as reported in chapter 2 and 3, the production levels of chemicals in the bulbs or roots also may have been affected. As a result, garlic seems to have developed high environmental adaptability so as to survive unfavorable climatic conditions.

(2) Roles of artificial and natural selection that may have caused differentiation in morphological, physiological, chemical, and genetic traits in garlic

In chapter 4, garlic showed various morphological, physiological, and isozyme variations among groups of accessions categorized according to their origins. According to chi-square tests, some loci deviated significantly ($p < 0.05$) from the Hardy–Weinberg equilibrium (HWE) (*Lap-1* and *Lap-2* loci in the Southeastern Mediterranean group and a *Lap-1* locus in the Southeast Asia group and Japan Group B). Other garlic research also observed deviated HWE using SSR markers (Ma et al., 2009; Jo et al., 2012; Garcia et al., 2012). It is believed that certain factors disturb the HWE (e.g., genetic drift, migration, natural or artificial selection, and non-random mating). Especially in garlic, it is predicted that this deviation can happen easily because this

plant reproduces clonally. However, the central Asia group, estimated to be the primary center of garlic origins, showed higher heterozygosity in isozyme loci than did other groups. Allelic frequencies also differed among groups. Garlic has specifically adapted to different agroclimatic regions (Figliuolo et al., 2001; Mario et al., 2008). There have been some reports of intraspecific variation among cultivated garlic landraces in Iran (Shaaf et al., 2014), Tunisia (Jabbes et al., 2012), Brazil (Buso et al., 2008), Argentina (Garcia et al., 2012), and China (Chen et al., 2013). These variations probably would be derived from standing variations or mutations caused by adaptation, hitchhiking, or genetic drift in the process of garlic's domestication before it was cultivated. It is likely that ancestral garlic populations would have had some standing variation in ancient times. When they expanded widely from their own growing fields to different agroclimatic regions, only adaptable clones survived. Alternatively, after the start of cultivation, as opposed to the variation resulting from sexual reproduction, it is expected that the variation of domesticated garlic might exist due to mutations accumulated through the history of cultivation (Shaaf et al., 2014). Therefore, there are two hypotheses: (1) domestication with some artificial selection occurred in Central Asia (these populations may have standing variations) and widely spread to other regions; or (2) domesticated garlic expanded to other regions of the world with accumulating mutations. In these cases, there are two possibilities: (1) the sources of local adaptation and artificial selection are derived from standing variations; or (2) the sources of local adaptation and artificial selection are derived from mutations accumulated during expansion. In other words, it is assumed that ancestral garlic populations have adapted in various regions using standing variations or mutations accumulated during expansion, evolving with human-preferred traits over a long history of cultivation.

(3) Potential of garlic collections as new breeding materials

Kamenetsky et al. (2005) stated that garlic from the place of origin possesses superior traits, such as tolerance to disease and pests and better adaptation to biotic or abiotic stress, than are seen in current cultivars. Our garlic from Central Asia produced many flowers but did not produce seeds. One of the main reasons is probably the growing condition. Etoh (1985) stated that the Mediterranean climate (cold in the winter, hot and dry in the summer) is suitable for growing garlic. Another reason is that garlic is maintained clonally. Long vegetative propagation, especially in garlic, resulted in widespread infection by viruses that cause yield reductions or stunted plant development (Conci et al., 2002). This fact suggests that virus infection impacts the formation of bulbs or bulbils or seed fertility. Therefore, it is necessary to treat bulbs for viruses in order to confirm the potential of a fertile garlic collection. Variations were observed in chemical as well as morphological traits. Chemical production levels were superior in some accessions, which would especially suit them for food and the health enhancement of food. Furthermore, as shown in chapter 3, some accessions produced many kinds of saponin compounds. This result suggests that it is possible that these accessions possess higher biotic stress tolerance than modern cultivars do. In addition, the garlic collections used in the present study also have possible abiotic stress tolerance of temperature or dryness. In chapter 5, we could determine high-adaptable garlic accessions. These accessions are able to open the new possibility of developing high-value-added garlic production in arid climate conditions.

In this study, a few genetic markers were used. If markers linked to agronomic traits were available, the rate of progress in breeding better garlic cultivars would

increase dramatically (Hong et al., 1997). Garlic breeding is hard work because garlic is a sterile plant. In addition, the size of a diploid garlic genome is approximately 15.9 Gbp, which is 32 times larger than that of rice (Ricroch et al., 2005). An Israeli garlic research group revealed more detail regarding the condition of fertile garlic (photoperiod, temperature, humidity, etc.) (Kamenetsky et al., 2001; Kamenetsky et al., 2004; Mathew et al., 2011; Shemesh et al., 2013). Recently, transcriptome analysis, a new approach with next-generation sequencing, is starting in various crops. Garlic researchers have also started to use this approach; Sun et al., (2012) and Kamenetsky et al., (2015) annotated 128,000, and 102,000 unigenes, respectively, that they obtained. Future garlic "omics" studies, including transcriptomes, will facilitate more helpful information such as DNA marker development and plant-pathogen interaction for future breeding programs (Kamenetsky et al., 2015).

On the other hand, the precious local gene pool is currently under severe threat of extinction, due to the rapid replacement of traditional landraces with modern cultivars of the *sativum* group (Ovesna et al., 2011; Kamenetsky et al., 2005). International world research institutes, such as the Plant Genetic Resources Institute (IPGRI) in Italy, the Volcani Center (ARO) in Israel, and the IPK in Germany, possess many types of genetic resources of garlic. Internationally, construction of an information structure for genetic resources of garlic should be imperative in near future.

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SUMMARY

Garlic (*Allium sativum* L.) which belongs to genus *Allium*, is vegetatively propagated plant. Garlic is important condiment vegetable which closely related to food culture in various regions. According to FAO, garlic is grown in various countries at a wide range of latitudes and with a total production of 24.8 million ton per year. Garlic is grown in various countries at a wide range of latitudes. The center of origin for garlic is considered to be the northwestern side of the Tien Shan Mountains, Central Asia. serious cultivation of garlic goes back approximately 3,000 BC in ancient Egypt. Today, large quantities of garlic bulbs are consumed as a functional food or for pharmaceutical purposes worldwide. Garlic which has primitive forms is considered as new breeding materials. In addition, there is a possibility that some garlic possesses superior traits, such as high chemical productions which contribute to human health, high tolerance to disease and pests, and high adaptation to biotic or abiotic stress, which never seen in current cultivars. The present study was conducted to evaluate the diversity of garlic worldwide, and to explore novel garlic breeding materials which have never seen in modern cultivars.

1) Characteristics of chemical components in genetic resources of garlic

Morphological observation of inflorescence was performed, and clones were divided into four types according to their bolting traits: Type A—bolters, producing mainly florets; Type B—bolters, producing mainly bulbils; Type C—incomplete bolters; and Type D—non-bolters. The appearance frequencies of the bolting types varied depending on the latitude of the collection sites. A comparison of these four different

types was carried out based on chemical composition data. It was confirmed that as distance increased from high-latitude areas (Central Asia and The Northern Mediterranean), the garlic was more likely to produce bulbils in the inflorescence with lower S-allyl-L-cysteine sulfoxide (AlCSO) content and higher phenolic content. This research suggested that garlic's transition from sexual propagation to asexual (vegetative) propagation and changes in the chemical composition of the bulbs would have occurred in the process of expanding garlic cultivation. In conclusion, garlic seems to have obtained high environmental adaptability with these transitions and changes via artificial selection.

2) Variations of saponin production in genetic resources of garlic collected worldwide

The production level of saponin components in 102 garlic accessions collected worldwide were determined. Quantitative analysis did not show the difference among collected regions but showed high CV scores among accessions. Meanwhile, the comparison between garlic and other related species in saponin spot profiling via thin-layer chromatography (TLC) showed variations among accessions. Garlic accessions have much diversity regarding the different kinds of saponins. Significant correlation between the geographical origins of accessions and saponin spots was not observed. These results suggested that garlic has adapted in various agroclimatic regions by producing unique saponin compounds over a long history of cultivation.

3) Evaluation based on the morphological, physiological, and isozyme variation in garlic collected worldwide

The morphological traits (bulb weight, bulb diameter, number of cloves per bulb, number of bulbils, and scape length) and a physiological trait (bolting period) of the garlic collected showed wide variations. Meanwhile, a total of 140 garlic accessions, including the 107 accessions mentioned above, were characterized by leucine aminopeptidase (LAP) and phosphoglucoisomerase (PGI) isozyme analyses; they clearly showed polymorphisms in putative isozyme loci (*Lap-1*, *Lap-2*, and *Pgi-1*). Allelic frequencies were estimated in each group of accessions categorized by their geographical origin, and the observed (H_o) and expected (H_e) heterozygosities were calculated. The allelic frequencies differed between groups. It was assumed that ancestral domesticated garlic populations have adapted in various regions using standing variation or mutations which accumulated during the expansion and it have evolved along with human-preferred traits over a long history of cultivation.

In this research, it was demonstrated that garlic from various regions has considerable variation not only in visible traits, but also in chemical production, or genetic level. This fact suggests that it is a possibility that these accessions possessing higher biotic stress tolerance than modern cultivars. In addition, these garlic collection used in the present study have also a possibility about abiotic stress tolerance such as temperature or dryness. Further research on the genetic structure of garlic populations is necessary to utilize new breeding materials for future marker-assisted garlic breeding programs.

JAPANESE SUMMARY

ニンニク（*Allium sativum* L.）はネギ科ネギ属の栄養繁殖植物であり，世界中の食文化に多大なる影響を与え続けている香辛野菜である．2013 年国際連合食糧農業機関（FAO）統計情報に見出せるネギ属野菜のデータをみると，約 2,480 万トンのニンニク総生産量はタマネギに次いで高く，経済的にも重要な種であることが窺える．起源中心地は天山山脈北西部の中央アジア地域とされ，5000 年以上の長い栽培の歴史をもち，栽培化が最初になされたと推定される古代エジプトでは既に人々の滋養と強壮に利用されていた．現在では，ニンニクは低緯度から高緯度までの幅広い緯度帯で栽培と利用がなされているが，栽培範囲が拡大していく過程で遭遇した様々な環境に適応するために，多様な遺伝的変異を蓄積していったと考えられる．起源中心地付近のニンニク系統の中には，抽苔，開花および結実を正常に行う稔性系統が多数発見されており，繁殖様式をより効率的な種子繁殖へ変換するための有望な育種素材とみなすことができる．また，稔性系統は既存の栽培種にはみられない土壌伝染性病害や高温・乾燥などの生物的ならびに非生物的ストレスに対する抵抗性を有している可能性が高い．そこで，本研究では，ニンニク遺伝資源から新たな育種素材を探索するために，世界に散在するニンニクの遺伝変異の様相を明らかにするとともに，その変異の中にみられる優良個体を選抜してニンニクの新たな育種素材としての可能性を検討した．本研究では，ニンニク系統が蓄積してきた突然変異の様相を明らかにするとともに，その変異の中にみられる優良個体を選抜して，ニンニクの新たな育種素材としての可能性を検討することを目的として以下の研究を行った．

(1) ニンニク遺伝資源の化学内容成分特性について

世界各地から収集したニンニク遺伝資源（103 系統）の慣行栽培を行い，抽苔特性を評価した結果，(a)：ニンニクは完全に抽苔し小花を形成するもの，(b)：完全に抽苔するが，

小花は形成しないもの，(c) ：抽苔が生育途中で停止するもの，(d)：全く抽苔しないもの，に分けることができた．また，形成した鱗茎の内容成分について，刺激臭の前駆物質であるアリルシステインスルホキシド（AlCSO）含量および機能性成分を含む総フェノールの含量をそれぞれ調査したところ，抽苔型ごとに含量の変化がみられた．すなわち，中央アジア地域由来のものに多く含まれる小花を形成するタイプでは，AlCSO 含量が高く，一方で，珠芽のみを形成するタイプは総フェノール含量が高くなっていた．このことから，花序の形態形成と鱗茎内化学成分に関連性があることが示唆された．ニンニクは各地へ伝播されていく過程において，可稔性から不稔性へ，そして完全抽苔型から不完全 - 非抽苔型へと様式を変化していったとされるが．本研究では，鱗茎内の化学成分組成を変化させて，高い環境適応力を獲得していったことがうかがえる新たな知見が得られた．

(2) ニンニク遺伝資源の根部におけるサポニン化合物生産について

ニンニク遺伝資源の根部における抗菌物質として知られるサポニン化合物の定量分析を実施したところ，系統間で含有量に違いがみられた．また，TLC（薄層クロマトグラフィー）によるサポニンの定性分析から，供試した系統において様々なサポニン化合物がみられ，広範な質的変異も確認された．これらのことから，ニンニクは，その長い栽培の歴史において生体内化学成分の特性に大きな変異が生じ，その変化を巧みに利用して異なる栽培環境に適応していったことが示唆された．

(3) ニンニク遺伝資源の形態・生理生態およびアイソザイム変異について

ニンニク遺伝資源の多様性を形態面から評価するために，上記と同様に栽培した植物体を用いて形態特性（花茎長，形成珠芽数，球重，球径，形成鱗片数および鱗片重量）ならびに生理生態特性（抽苔するまでの日数）に関する調査を行った．また，日本産を加えた 140 系統の遺伝資源を用いてアイソザイム分析を行い，集団遺伝学的解析を駆使してニ

ンニク酵素多型の由来を検討した．形態および生理生態特性に関しては，花茎長や球重量などの特性について収集地域間で有意な差が認められた．アイソザイム分析の結果，LAP および PGI 由来の遺伝子座（*Lap-1*，*Lap-2* および *Pgi-1*）において明瞭な多型がみられた．これら遺伝子座に関して，各収集地域における対立遺伝子の頻度を調査したところ，収集地域間で出現頻度に違いがみられることがわかった．また，ヘテロ接合度を算出したところ，起源地である中央アジアや地中海地域では高く，その他の地域では低くなるという傾向がそれぞれ得られた．収集地域間の分化の程度を示す遺伝的分化指数（GST）は 0.259 となり，地域間でかなりの分化が進んでいることが示唆された．また，地中海南東部，東南アジアおよび日本の諸島由来の系統は有意にハーディー・ワインベルグ平衡（HWE）HWE から逸脱する現象がみられ，これらの地域では HWE を乱す因子（集団間の移動，遺伝子流動，自然あるいは人為選抜など）が存在したと推察された．以上の結果から，ニンニクが起源中心地から生育範囲を拡大していく過程において，祖先集団があらかじめもっていた変異（standing variation）と拡大過程におけて蓄積していった突然変異の相乗効果により環境適応性が向上し，さらに，各地で抽苔や球肥大などの特性に関する人為的な選抜が行われたことで進化の方向性が栽培化へ向けて進んでいった変遷が示された．

本研究により得られた新たな知見により，ニンニク遺伝資源の形態，生理生態，化学内容成分ならびに酵素遺伝子に関する広範な多様性が見出された．それらの中には機能性成分の生産能が高い系統や生物的・非生物的ストレスに対する抵抗性をもつことが期待される系統が含まれていた．これらについては，今後，ニンニクの新たな育種素材として活用することが期待される．

LIST OF PAPERS RELATED TO THE THESIS

Hirata, S., Abdelrahman, M., Yamauchi, N., and Shigyo, M. 2015. Characteristics of chemical components in genetic resources of garlic *Allium sativum* collected from all over the world. Genet. Resour. Crop. Evol. DOI 10.1007/s10722-015-0233-7. (In press).

(In relation to Chapter 2)

Hirata, S., Abdelrahman, M., Yamauchi, N., and Shigyo, M. 2015. Diversity evaluation based on the morphological, physiological, and isozyme variation in genetic resources of garlic (*Allium sativum* L.) collected worldwide. Genes. Genet. Syst. (In press).

(In relation to Chapter 4)

ACKNOWLEDGEMENT

I wish to express my sincerest thanks to Dr. Masayoshi Shigyo, my major supervisor (Professor of the Department of Biological and Environmental Sciences, Faculty of Agriculture, Yamaguchi University, Japan) for his scientific guidance. He always provides me with invaluable suggestions for my scientific research. I greatly appreciated his encouragement for my study and advice for my daily life throughout the time I have studied in the Laboratory of Vegetable Crop Science, Yamaguchi University.

I would like to express my deep gratitude to Dr. Naoki Yamauchi (Professor of the Department of Biological and Environmental Sciences, Faculty of Agriculture, Yamaguchi University, Japan) for his kind support and encouragement for my work.

I am very grateful to Dr. Nobuo Kobayashi (Professor of Faculty of Life and Environmental Science, Department of Agriculture, Shimane University, Japan) for his invaluable advice and suggestions regarding my study.

I wish to thank Dr. Takeomi Etoh (Professor Emeritus of Kagoshima University, Japan) for providing garlic materials collected worldwide, including in Central Asian regions.

I am very grateful to Dr. Yosuke Tashiro (Professor Emeritus of Saga University, Japan) for providing garlic materials collected from a wide area in Japan.

I also thank our laboratory members, my friends, and my family most sincerely for supporting me intellectually.